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MOUSE CYTOMEGALOVIRUS INFECTION
AS A MODEL FOR
PERSISTENT VIRAL INFECTIONS
IN MICE AND HUMANS

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September 2005

**Submitted to fulfil the requirements for the degree of Doctor of
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dedicated to my wife Annette

and

to my parents

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1. Summary

Background: CD8⁺ T cells are critical not only for the termination of acute viral infections but also for the long-term control of viruses capable to establish persistent infection such as Human Immunodeficiency Virus (HIV), Hepatitis B and C virus (HBV and HCV), Epstein Barr Virus (EBV) and Cytomegalovirus (CMV). The level of viral persistence (or antigen load) has an important impact on the magnitude and the quality of virus-specific T cells and vice versa. Usually, high antigen load during a prolonged period of time has a damaging influence on the functionality of antiviral T cell responses. However, it is less clear whether low level antigen persistence is beneficial or detrimental for the maintenance of virus specific T cells and ultimately for protective T cell memory. This has obvious implications for vaccine design.

Hypothesis: I speculated that antigen persistence at a low level is beneficial for the long-term maintenance of virus specific CD8⁺ T cells and for their protective capacity against challenge infections.

Methods: Mice were infected with mouse cytomegalovirus (MCMV), which leads to a low level persistent or latent infection with the potential for viral reactivation. To compare the long-term dynamics of virus specific CD8⁺ T cells after persistent (MCMV) or transient infections [Vaccinia virus (VV), Influenza A virus (Influenza)], I used recombinant MCMV expressing T cell epitopes derived from heterologous viruses. Thereafter, I longitudinally characterized the magnitude, the phenotype and the

functionality of CD8⁺ T cells specific for native and recombinant epitopes in detail and tested their long-term protective capacity against viral challenge.

Results: This longitudinal analysis of the native MCMV-specific CD8⁺ T cell response revealed a most unusual pattern of T cell dynamics: after initial expansion and limited contraction MCMV-specific memory CD8⁺ T cells steadily accumulated over time, with 20% of all CD8⁺ T cells being specific for a single MCMV-derived T cell epitope at one year after infection. Accumulation of effector memory T cells was seen in all organs tested and was accompanied by a gradual restriction in T cell receptor V β chain usage over time. The pattern of accumulation, which I have termed ‘memory inflation’, was observed only in two out of five epitopes tested. Responses against other tested epitopes followed more ‘classical’ dynamics with initial expansion and contraction to stable memory T cell levels.

Challenge with recombinant VV more than 6 months after MCMV-infection provoked vigorous expansion of these ‘inflated’ effector memory T cells, which mediated immediate protection against challenge. Accumulation of effector memory T cells was also seen after infection with recombinant MCMV and long-term protective immunity, based on recombinant T cell epitopes, was maintained more efficiently than after transient infection with non-persisting viruses, which expressed identical epitopes.

Conclusions: My results have two major implications: 1) ‘Memory inflation’ is a previously unrecognised pattern of an antiviral T cell response, which is most likely driven by continuous or repetitive antigen encounter during latent or low-level persistent MCMV-infection. Recent cross-sectional studies in humans suggest that similar virus-

host interactions might exist for human viral infections particularly with CMV and EBV.

2) Effector memory T cells accumulating during low-level persistent infection provide efficient long-term protective T cell immunity without the need for boosting. These results demonstrate that low-level antigen persistence is beneficial for protective T cell memory and they provide the first 'proof-of-principle' evidence to encourage further exploration of persistent 'self-boosting' vectors for the development of successful T cell based vaccines.

2. General Introduction

2.1. Persistent infections

Persistent infections of humans are an enormous problem both for the affected individual and for human society in general. Infection with human immunodeficiency virus (HIV) and subsequent acquired immune-deficiency syndrome (AIDS) has become the most prominent cause of death for adults in sub-Saharan Africa and infection rates are still rising, reaching up to 40% of working age people in some areas. By December 2003 WHO estimated that about 40 Mio people are currently infected with this persistent virus and that per day 14'000 new infections occur worldwide (1).

Furthermore, other persistent human pathogens including viruses, bacteria and parasites, which have plagued the human race for much longer than HIV are far from under control. Tuberculosis and leprosy are the most prominent bacterial examples, viruses include hepatitis B and C virus (HBV and HCV) and all of the herpesviruses. Parasitic infections like trypanosomiasis, some forms of malaria and toxoplasmosis can establish persistent infection as well. However, many of these infections can be controlled by the host's immune system for a very long time and sometimes for life without overt disease. Some of these organisms have reached a remarkable state of more or less peaceful coexistence with their host during evolution. Since seroprevalence for these infections can reach up to 95%, coinfection and superinfection with HIV is now frequently encountered. With increasing immunosuppression during the development of AIDS, immune control over these pathogens is lost leading to disease and death. Reactivation of persistent infections poses an additional problem in modern medicine as a

complication of therapeutical immuno-suppression during organ transplantation and treatment of cancer, leukaemia and autoimmune disease.

Therefore, our understanding of persistent infections and of the factors influencing their control within individuals and within the susceptible population is highly relevant for their prevention and treatment. Understanding the interactions of these infectious organisms with the immune system of the host is central for the rational development of immune based prevention (prophylactic vaccines) and treatment strategies (therapeutic vaccines and immunotherapy).

The direct manipulation of an infectious disease and of the ensuing immune response in humans is often not possible. Moreover, in vitro studies cannot imitate the complexities of two biological systems in a dynamic interaction, i.e. virus infection, dissemination and establishment of latency versus a complex immune response with various effector mechanisms. Particularly the geographical and sequential variety and complexity of events, which take place during the course of an infection of a vertebrate host with a pathogen, are beyond the scope of in vitro systems. Therefore, animal models have been used for decades to study infections and their consequences for the host. However, whether basic principles established in animal models apply for infections in humans has to be tested individually before general conclusions can be drawn.

Mice have been the study animal species of choice for immunology during the last 50 years. Thus, we know more about mouse immunology than of any other species except humans. However, only few mouse models have been developed for the study of persistent viral infections, but they have contributed tremendously to our understanding of successful and failing immune control of persistent infections.

The following studies of mouse cytomegalovirus (MCMV) infection were undertaken to learn more about the immune response against persistent viruses in general and to further characterize a well established animal model of an important human pathogen with current immunological techniques.

2.2. Human and mouse cytomegalovirus

Cytomegaloviruses (CMV) belong to the family of β -herpesviruses. They share many biologic features with other herpesviruses including virion and genome structure, and the ability to establish persistent and latent infection. Since they have coevolved with their hosts for millions of years, they are highly species specific and many different animal species have their 'private' CMV. Infection across species barriers is usually not possible (2).

2.2.1. Epidemiology and clinical symptoms

CMVs are evolutionary very successful and they are highly adapted to their respective host. In humans, 60-90% of the adult population are healthy, seropositive carriers of human (H) CMV. Most people acquire infection before puberty: up to 2% are infected during gestation, another 8-60% during the first 6 months of life (during birth, by breast feeding) and about 20-80% during the next five years mainly by close contact to infected children (nurseries). Sexual transmission represents another major route of infection in adulthood (3).

Primary HCMV-infection in immunocompetent individuals is usually clinically silent. Rarely, a self-limiting mononucleosis-like syndrome with fever, myalgias, splenomegaly and abnormal liver function tests can be attributed to acute HCMV-infection. More

problematic are primary infections during pregnancy with vertical transmission. Congenital HCMV-infection with a mortality rate of about 10% is characterized by transient jaundice, hepatosplenomegaly and thrombocytopenia and by persistent neurological defects and/or mental retardation in up to 80% of symptomatic cases (3). Primary HCMV-infection and more frequently reactivation of latent virus in immunocompromized individuals represent a second important clinical problem. Transplant recipients (bone marrow (BM), and solid organs) and AIDS-patients (particularly those with CD4⁺ T cell counts below 50 cells/ μ l) suffer from various manifestations of HCMV-disease including central nervous system infection and chorio-retinitis, gastrointestinal and hepatobiliary syndromes and life-threatening pulmonary infection (3). Availability of antiviral drugs both against HCMV and HIV has contributed to a more favourable outcome of CMV-disease in immunocompromized patients. However, this achievement is restricted to developed countries where antiviral drugs are affordable.

2.2.2. Genome of Cytomegaloviruses

HCMV does not share significant nucleotide sequence homology with other CMVs including MCMV except for enhancer and genome packaging sequences (4). However, there is a far-reaching functional overlap between HCMV- and MCMV-derived proteins. Thus, positional and functional homologues exist between these two CMV (2). HCMV and MCMV share a similar genomic organization: they have a large (230-235kB) double-stranded, linear DNA-genome encoding > 200 open reading frames (ORF) (5). A core of genes, which are common to all herpesviruses, is arranged as gene blocks in the central 100kB of the CMV-genome. Mainly 'house keeping' functions are encoded in

these gene blocks including DNA-replication and repair, nucleotide metabolism or virion structure (2).

CMV-replication is tightly regulated in a multistep process, which is subdivided into immediate-early (IE), early (E) and late (L) phase of gene expression. IE-gene expression can be detected within 20-30 min after exposure of cells to virus (6). Two major gene products are transcribed from the IE-locus: IE1 and IE2 in HCMV and IE1 and IE3 in MCMV. In MCMV, a third gene product of the IE-locus, called IE2, is transcribed in the opposite direction. MCMV-IE2 whose function is unknown has been shown to be dispensable for in vitro and in vivo viral replication (7). Productive virus replication requires the expression of first IE1 and then IE2 (HCMV) or IE3 (MCMV), respectively, since these gene products specify a crucial transactivator necessary for the expression E- and L-phase genes (8). During MCMV latency, transcription of IE1 and somewhat less frequently of IE2 has been detected without evidence for full reactivation which is defined by the emergence of infective viral particles (8-10). This probably reflects the sporadic and intrinsic activity of the enhancer element controlling the IE1 and IE2 promoter (10). IE3 however, was not transcribed during latency but was only detectable if reactivation was promoted by immunosuppression or allogeneic stimulation (8, 11).

2.2.3. Virion structure

Mature virions of CMV consist of an icosahedral capsid of about 100nm diameter that is surrounded by a tegument or matrix. The capsid contains the viral genome, which is closely associated with core proteins forming the nucleocapsid. In addition, mature virions of 150-200nm have a host cell derived lipid bilayer envelope with several virally encoded glycoproteins (gp) (12). In tissue culture, cells infected with HCMV produce a

substantial amount of defective viral particles called 'dense bodies' (DB), which lack a nucleocapsid and are largely composed of abundant tegument phospho-protein (pp) pp65/ppUL83 (pp65) with an envelope (13). In contrast, MCMV does not produce DB but has a propensity for multi-capsid-virions (14).

2.2.4. Viral proteins

2.2.4.1. Structural proteins

The HCMV-capsid is mainly composed of the major capsid protein (MCP/pUL86) and the minor capsid protein (mCP/pUL46), both exhibit low immunogenicity (15). The tegument proteins including pp150/ppUL32 and particularly pp65 are immunologically more interesting since they are more immunogenic. Both elicit a readily detectable but not neutralizing antibody (Ab) response and have thus been used for serodiagnostic purposes (16, 17). pp65 is highly abundant in the tegument of intact virions and even more so in DB which consist to 95% of pp65 (18). The exact function of pp65 is not clear yet but it was proposed to have an immune evasive function by inhibiting proteasomal processing of other HCMV-derived proteins like IE1 (19). It is now clear that pp65 is a major target of the HCMV-specific CD8⁺ cytotoxic T cell (CTL) response in persistently infected asymptomatic individuals (20-23) and several CTL-epitopes with differing restriction by the major histocompatibility complex (MHC) have been identified (24). CD8⁺ T cells specific for the other major tegument protein ppUL32/pp150 of HCMV have also been identified but they are much rarer than pp65-specific cells (25).

In mice, CD8⁺ T cells specific for the positional and functional homologues of pp65 namely ppM83 and pM84 have been identified after MCMV-infection. However, the response seems to be much less prominent compared to pp65 in humans (26, 27).

Virally encoded envelope glycoproteins (gp) are the major targets of the host's neutralizing Ab-response in mice and men (15, 28). The major envelope gp of HCMV gB/gpUL55 (gB) plays a role in virion penetration of cells, transmission from cell to cell and fusion of infected cells (29). gB from HCMV and MCMV share extensive amino acid identity and antigenic crossreactivity (2, 30). Neutralizing Ab directed against gB can block all of the above mentioned functions of gB but neutralization is usually complement (C) dependent (31). HCMV-gB-derived epitopes for CD8⁺ and CD4⁺ T cells have also been described (25, 32, 33). C-independent neutralizing Ab are mainly directed against a second HCMV-derived envelope glycoprotein called gH/gpUL75, which is involved in membrane fusion and cell-to-cell transmission of virus (34). There is little AA-sequence variation between different CMV-isolates within envelope gp (35). Therefore, most of the neutralizing Ab-epitopes of gB and gH are conserved and different serotypes have not been defined, although minor variations exist.

2.2.4.2. Regulatory proteins

Non structural regulatory CMV-proteins are highly relevant to understand the biology of the virus and its interactions with the host. CMVs are slowly replicating viruses with a rigid dsDNA-genome, which encode an estimate of up to 200 proteins. Therefore, they seem to be an easy prey for the host's immune system. However, like other herpesviruses they have developed sophisticated strategies to prevent complete elimination by the immune system and to persist permanently for the lifetime of the host (2). The most

important strategy of CMVs to persist is to hide from the immune system by latency. In latency, viral proteins, which are the major target for the immune system, are not produced (or only to a very limited extent). For transmission to a new host viral proteins have to be expressed and viral particles produced which then represent a target for the immune system. To limit this, CMVs encode a whole arsenal of genes interfering with the antiviral immune response (36).

Some of the important regulatory proteins of CMV replication and latency are encoded during the IE-phase of virus replication. HCMV-IE1/pp72 and MCMV-IE1/pp89 are major targets of the host's antiviral CD8⁺ T cell response since they are usually expressed before the immune evasive genes become operational, which are mostly encoded during the E-phase of viral replication (25, 32, 37, 38).

In recent years many functions of immune evasive proteins of CMV have been characterized on a molecular level. One of their major targets is the MHC class I Ag-presentation pathway. In MCMV, mainly three different proteins interfere profoundly with the cell's Ag-presentation machinery. M06 binds to peptide-loaded MHC class I molecules in the endoplasmatic reticulum (ER) and redirects them to an endosomal/lysosomal compartment for degradation (39). M152 triggers the retention of peptide loaded MHC class I complexes in an ER/Golgi intermediate compartment (40, 41) and m04/gp34 escorts MHC class I complexes to the cell surface but seems to prevent their recognition by CD8⁺ T cells (42). It has been speculated that this might serve to silence NK-cells alerted by the lack of MHC class I expression on the surface of MCMV-infected cells (43).

In HCMV, US6 blocks peptide translocation through the transporter associated with Ag-presentation (TAP) (44, 45). US3, which is the only immune evasive protein expressed

during the IE-phase of HCMV-replication, causes retention of MHC class I peptide complexes in the ER, which are then degraded by US2- and/or US11-mediated mechanisms (46). In addition, US2 and US11 can mediate retrograde translocation of MHC class I molecules from the ER to the cytosol where they are rapidly degraded by the proteasome (47, 48).

This profound downregulation of MHC class I molecules on the surface of infected cells could render these cells particularly vulnerable to NK-cell mediated destruction, which are activated by the 'missing self' (49). However, CMVs seem to have evolved additional strategies to avoid NK-cell mediated elimination (50). As an example, MCMV encodes the MHC class I homologue m144, which has been shown to inhibit NK-cells mediated clearance of infected cells (51). Moreover, HCMV-derived UL40 was shown to upregulate surface expression of HLA-E, a non classical MHC-molecule which is able to silence NK-cells (52).

2.2.5. Immune response after CMV-infection

Since CMV-infection does only lead to substantial morbidity and mortality in immunocompromised individuals, the immune response must be critically important in the control of overt disease. On the other hand we know that protective immunity is incomplete and viral persistence is invariably established upon primary infection. A delicate balance between virus and host has co-evolved permitting the survival and reproduction of both.

Most primary infections with CMV do not provoke sufficient clinical symptoms for patients to seek medical support. Therefore, these events are usually not recognized particularly during childhood. Some individuals develop a mononucleosis-like disease

resembling acute Epstein Barr virus (EBV) infection with fever, lymphadenopathy, lymphocytosis and moderately elevated liver function tests (53). Probably, these symptomatic patients represent a selected population, where either the replication of CMV or the resulting immune response (or both) are more intense than in asymptomatic infection (54, 55). However, our knowledge about early events in HCMV-infection of immunocompetent individuals has been mainly gathered from studies of these symptomatic patients.

Transmission of CMV occurs by exchange of or exposure to blood and other body fluids like saliva, breast milk, urine and genital secretions. The incubation time from exposure to the onset of symptoms varies between 20-60 days. By then, virus is usually detectable in blood, pharyngeal swabs and urine. Plasma viremia is transient and below detectable levels within 2-3 weeks whereas pp65-antigenemia and particularly leukocyte associated viral DNA remains detectable for several months (56). Infectious virus can be recovered for several weeks from pharyngeal swabs and for up to one year from urine. HCMV excretion may be even substantially longer in children infected below 2 years of age (57, 58).

Signs of a vigorous immune response during primary HCMV-infection of symptomatic patients are obvious on clinical examination (fever, lymphadenopathy, splenomegaly) and on a simple blood smear (lymphocytosis with 'atypical' cells). Most of these 'atypical' lymphocytes belong to the CD8⁺ T cell compartment (59). Because of similarities to acute EBV-infection, where massive expansions of EBV-specific cytotoxic T cells (CTL) were demonstrated (60), it is speculated that these cells are mainly HCMV-specific CTL. However, a detailed analysis of the exact nature,

magnitude, specificity and kinetics of the antiviral immune response during primary HCMV-infection has not been performed in immunocompetent individuals.

2.2.5.1. Innate immunity

In analogy to other viral infections and particularly because of data from the MCMV-model, innate immunity has been implicated in the control of primary HCMV-infection. Interest has focussed on natural killer (NK) cells since HCMV and MCMV encode several proteins with the capacity to interfere with NK cell activation (61, 62). Therefore, it seems likely that NK cells have exerted an evolutionary pressure on these viruses. In addition, patients with NK cell deficiencies concerning both number and function often develop severe infections with herpes viruses including HCMV (63, 64). However, direct evidence is limited concerning the exact role of NK cells in primary HCMV-infection and whether they form an important 'first line of defence' remains speculative.

In MCMV-infection, work from the group of R. Welsh suggested that NK cells limit early virus replication. Depletion of NK cells led to increased mortality due to unrestricted viral replication and dissemination (65). Analysis of resistant and susceptible strains of inbred and congenic mice led to the identification of an activating natural killer receptor called Ly49H which confers resistance to MCMV (66). Both direct cytotoxicity and interferon (IFN) γ release of NK cells participate in the termination of productive MCMV-replication in an organ specific manner (67). In addition, NK cells together with T cells help to prevent MCMV-reactivation and thus maintain latency in a hierarchical and redundant fashion (68).

The role of other cells of the innate immune system in HCMV-infection is less clear. Neutrophils are probably of minor importance since neutropenic individuals are not primarily prone to viral infections unless their lymphocyte number or function is suppressed as well (69). Macrophages seem to have a dual role: first, they are targets for virus replication and dissemination into tissues; second, they clearly exhibit antiviral functions both directly by the release of antiviral cytokines and chemokines and by activation of adaptive immune mechanisms (70). In MCMV their overall role in resistance is probably beneficial for the host whereas in HCMV this remains unclear since crucial experiments cannot be performed for obvious reasons (71).

Of all the different innate immune mechanisms based on soluble proteins, type I ($\alpha\beta$) and type II (γ) IFN seem to have the largest impact on CMV-replication, although clinical trials evaluating the effect of IFN γ and IFN $\alpha\beta$ in the prevention or treatment of HCMV-infection have demonstrated very limited clinical benefit and a modest antiviral effect (72, 73). However, mice depleted of IFN in vivo or deficient for IFN receptors showed increased susceptibility after MCMV-infection (74). Particularly IFN $\alpha\beta$ seems to be essential for early containment of viral replication and survival since receptor knock out animals died within 5 days after infection (75). IFN γ -receptor deficient animals also show increased early susceptibility to MCMV-infection but they usually survive for several months but then develop lethal inflammation of the great vessels (76, 77)).

Members of the tumour necrosis factor (TNF) family of proteins have a distinct influence on CMV replication. For HCMV, this was mainly analysed in vitro. While exogenous TNF α did not inhibit HCMV-replication in fibroblasts, lymphotoxin (LT)- α had a profound influence abolishing productive infection completely by increasing the

production of IFN β (78). This seems to be similar in mice, since neutralization of TNF α or deficiency in the TNF-receptor 1 had only a minor influence on MCMV-replication (79, 80). In contrast, LT α -deficient mice were highly susceptible to MCMV-infection due to inefficient induction of IFN β -production (78).

Although the complement system is important for the action of CMV neutralizing antibodies in vitro there is no indication that this system is crucially involved in CMV control in vivo. However, as yet another immune evasive strategy of MCMV the membrane regulator of complement CD46 is upregulated in MCMV-infected cells leading to their protection from complement mediated lysis (81).

2.2.5.2. Adaptive immunity

Overall, the most important cells in immune control of CMV-infection are CD8⁺ T cells. Recovery from HCMV-disease coincides with emergence of virus specific CD8⁺ T cells in recipients of allogeneic bone marrow (82, 83). Both in humans and in mice adoptive transfer of CMV-specific CD8⁺ T cells was sufficient to terminate productive virus replication and thus prevent CMV-disease and death in otherwise immunodeficient hosts (84, 85). In addition, using recently developed techniques to monitor T cell responses, large populations of HCMV-specific CD8⁺ T cells have been identified in asymptomatic seropositive individuals (21, 86-88). The size of these populations, which often reach several percentages of the total CD8⁺ T cell-pool, suggest an active involvement in long term control of the virus. However, in HCMV-infected young children, who shed infectious virus in urine and saliva for more than a year, HCMV-specific CD8⁺ T cells were found in similar frequencies compared to adults without evidence for productive

viral replication (58). It has been speculated that CD8⁺ T cells might be preferentially responsible to limit systemic but not peripheral HCMV-replication.

The HCMV-specific CD8⁺ T cell-responses are mainly targeting two immunodominant proteins: the structural matrix protein pp65 and the regulatory protein IE1/pp72 (24).

However, in contrast to EBV-infection, we completely lack data on the longitudinal evolution of HCMV-specific T cell responses in immunocompetent individuals, starting from primary infection. Therefore, we can only speculate how these responses develop.

In MCMV-infection, robust CD8⁺ T cell responses developing within 7-10 days have been described in detail by Reddehase and Koszinowski (89-91). Using classical ⁵¹Cr-release and limiting dilution assays after in vitro restimulation to analyse cellular cytotoxicity, they could demonstrate CTL-activity from day 6 until several months after infection, when productive replication was long terminated. Dependent on the mouse strain analysed peptides from different proteins were shown to be immunodominant including one H2-D^d-restricted epitope derived from IE1/pp89 (pp89). MCMV-homologues of the HCMV matrix protein pp65, which is immunodominant in humans, were clearly subdominant epitopes (24). Particularly in the BALB/c mouse strain, where the antiviral activity of NK cells is less dominant, CD8⁺ T cells have been shown to efficiently terminate productive infection. Furthermore, they were shown to be important for the maintenance of latency and the prevention of MCMV-reactivation, although NK cells and CD4⁺ T cells were able to compensate their absence (68).

A direct antiviral effect of CD4⁺ T cells is less well established and is mainly derived from studies in mice. In contrast to CD8⁺ T cells, adoptive transfer of CD4⁺ T cells from MCMV-infected immunocompetent mice failed to protect immunosuppressed animals against lethal MCMV-challenge (92, 93). However, in a situation of CD8⁺ T cell-

deficiency primary infection with MCMV is cleared with similar efficacy by CD4⁺ T cells and NK cells and this protection against disease and death can be adoptively transferred to new hosts (94, 95). In addition, CD4⁺ T cells seem to have a unique role in the termination of productive viral infection in salivary glands and this antiviral effect is mediated by IFN γ (96, 97).

In humans we know very little about the role of CD4⁺ T cells in immunity against HCMV. Substantial populations of HCMV-specific CD4⁺ T cells have been described in latently infected individuals but again we lack data on primary infection of immunocompetent individuals and their longitudinal follow up (98-100). In HCMV-infected young children shedding infectious virus in urine and saliva for more than a year, HCMV-specific CD4⁺ T cells were shown to secrete substantially less IFN γ and IL-2 (101). However, whether this is an underlying reason for or a consequence of persistent viral replication remains unclear. In HIV-infected individuals with severe immunosuppression particularly those with a CD4⁺ T cell-count < 50 cells/ μ l, HCMV-reactivation is one of the major opportunistic infections and has been classified as an AIDS-defining disease. Since the number of CD4⁺ T cells in the blood correlates negatively with the risk for HCMV-disease, an important role for CD4⁺ T cells to prevent reactivation has been postulated (102). Furthermore, in HCMV-seronegative immunosuppressed recipients of a solid organ from a HCMV-seropositive donor, appearance of virus specific CD4⁺ T cells correlated better with disease resolution than reconstitution of CD8⁺ T cells (103, 104). However, it remains to be elucidated whether these are direct antiviral effects of CD4⁺ T cells or whether they function mainly by interacting with other cells, particularly CD8⁺ T cells.

Both neutralizing and binding antibodies can be detected within weeks of primary infection with HCMV and neutralizing antibodies are mainly directed at the envelope GP gB and gH (15, 28). Most of their neutralizing activity is complement dependent (2). A role for neutralizing antibodies in the limitation of CMV-disease is suggested by several observations: first, preconceptional immunity usually protects newborns from severe infection although not completely (105-107). Second, vertical transmission of the virus was less frequent in women with high titres of neutralizing antibodies (108). Third, the serum level of HCMV-neutralizing antibodies was inversely correlated with progression of CMV-retinitis in AIDS patients (109). Forth, clearance of HCMV-antigen from the blood was faster in those solid organ transplant recipients that produced high avidity antibodies (110). Last, survival of active HCMV-infection in allogeneic bone marrow recipients was associated with the level of anti-gB antibodies (111). However, some of these findings could also be explained by differences in cell-mediated immunity particularly concerning CD4⁺ T cells providing cognate help for HCMV-specific antibody responses.

In mice, antibodies were dispensable for clearance of primary infection but they were able to limit the systemic spread of recurrent MCMV-infection (112). Overall, neutralizing antibodies are probably most important during gestation and the early neonatal period to protect progeny from severe CMV-disease.

2.3. T cell responses during transient and persistent viral infections

Recent technological advances in the quantitative analyses of virus specific T cell responses have enabled us to quantify, monitor and analyse human T cells directly *ex vivo*. Studies using these methods have confirmed some earlier results, dismissed others

and helped to generate several new concepts of T cell biology and homeostasis. Intracellular cytokine staining (ICS), enzyme linked immunospot assay (ELISpot) and major histocompatibility complex (MHC) class I tetrameric complexes (tetramer) have all been used to measure epitope specific T cells more accurately than with older techniques i.e. ^{51}Cr -release assay and limiting dilution analysis (see Chapter materials and methods for detail) (113). Particularly the development of tetramer and the subsequent analysis of the phenotype and the function of epitope specific CD8^+ T cells directly ex vivo has had an enormous impact on our understanding of virus specific immune responses (114, 115).

2.3.1. Bystander activation of T cells

It has been known for several decades that massive expansions of lymphocytes occur during acute infections. Most of these cells were assumed to be activated as ‘bystanders’ since their specificity could not be attributed to the infecting pathogen (116-118). However, the oligoclonal origin of these T cells in certain acute viral infections had already suggested that these expansions were dependent on interaction of the T cell receptor (TCR) with specific antigen and not driven by some unspecific ‘bystander’ signal (60, 119). This has been confirmed by studies using ICS, ELISpot and tetramer both in murine and human viral infections (120-123). In some acute viral infections like EBV in man or lymphocytic choriomeningitis virus (LCMV) in mice up to 50% of all CD8^+ T cells can be specific for a single viral epitope. Overall, the magnitude of CD8^+ T cell responses in viral infections measured with these new and more sensitive methods is about 10-fold larger than previously estimated (124). Therefore, the concept of bystander

activation as an important factor in antiviral immune responses has been largely abandoned.

2.3.2 Clonal burst size and T cell memory

For transient infections, studies using tetramers confirmed a model that predicts the size of the memory T cell pool being dependent on the initial burst size of the response: the 'burst size model' (120, 125). Usually, after initial expansion of effector T cells and subsequent clearance of the virus, the frequency of specific T cells drops and is then stabilized at a level of about 5-10% of the maximal frequency of T cells during the acute response. Although this general rule has been generally confirmed, the quantitative analysis of antiviral T cell responses with newer techniques revealed frequencies which are about 10 times higher than what was estimated with older measurements both during acute infection and also in memory (120, 126). Not surprisingly, there are certain exceptions: in persistent infections epitope specific T cells persist at higher frequencies than anticipated from the size of the primary response (126, 127).

2.3.3. T cell responses against viruses that persist at high levels

Studies of persistent infections in mice and in humans have revealed that the amount of virus persisting in a host has a fundamental influence on the resulting T cell response. Examples of high level persistence like chronic HIV- or HCV-infection in humans and certain virus isolates of lymphocytic choriomeningitis virus (LCMV)-infection in mice, as well as examples of low level persistence like EBV- or HCMV-infection in humans and murine γ -herpesvirus 68 (MHV-68) or MCMV-infection in mice will be discussed in the following section.

The level of persistence is obviously dependent on the biology of the virus. Viruses with a high cytopathogenicity cannot persist at a high level for prolonged time periods without killing the host, and thus jeopardizing their own survival. Therefore, nearly all viruses capable of high level persistence are poorly cytopathic or, as an exception, they infect cells that are not required for host survival (128). In addition, most viruses capable of high-level persistence have a rather small genome and a high replication rate. Overall, their strategy to persist despite the defence mechanisms of the host is one of 'speed' and 'shape change' (129). Nevertheless, the immune response seems to have an important impact on the outcome of these infections.

2.3.3.1. T cell responses in HIV-infection

HIV mainly infects activated CD4⁺ T cells via CD4 and a co-receptor (130-132). During the course of the infection CD4⁺ T cells are gradually lost and progressive immunodeficiency develops in infected individuals. The most important clinical parameters predicting the prognosis of an HIV-infected individual after resolution of primary infection are the CD4-count and the 'set point' virus load (VL): the probability of disease free survival increases with higher CD4-counts and lower VL (133). However, in the natural course of infection nearly all infected people develop AIDS, which is characterized by an increased susceptibility to opportunistic infections and tumours. It was recognized early on that a strong antiviral immune response was active during all phases of HIV-infection. An important role for HIV-specific CD8⁺ T cells has been suggested by several different studies over time. First, it was noted that the appearance of HIV-specific CD8⁺ T cells during primary HIV-infection was associated with a drop in viremia to the 'set-point' VL (119, 134, 135). In chronic infection the level of CTL-

reactivity was inversely correlated with VL, CD4⁺ T cell-decline and disease progression (136). In addition, HIV-specific CTL seem to exert an important evolutionary pressure on the virus, since CTL-escape mutations frequently occur in different phases of the infection, and they can be associated with loss of immune control (137, 138). However, the most convincing evidence for CD8⁺ T cell-mediated immune control of HIV comes from experiments performed in macaques infected with the simian immunodeficiency virus (SIV), which is closely related to HIV. Upon in vivo depletion of CD8⁺ T cells with monoclonal antibodies, a sharp rise in SIV viremia and a rapid progression to simian AIDS was observed (139, 140).

After identification of large populations of specific CD8⁺ T cells both in acute and chronic HIV-infection, the question arises, why these cells cannot control the virus in the long term in most infected individuals. Many different mechanisms have been postulated and some have been demonstrated experimentally, but usually in a restricted set of individuals (reviewed in (141)): 1) Viral escape by selection of mutations of immunodominant CTL-epitopes and subsequent loss of immune control was recognized early on in different phases of the disease course and has since been repetitively confirmed (137, 142). 2) Maintenance of CTL-activity in persistent infections is probably dependent on virus specific CD4⁺ T helper cells, and they seem to be preferentially infected by HIV and possibly lost already during primary infection (143-145). 3) HIV-specific CD8⁺ T cells might be deleted in phases of high viral replication due to clonal exhaustion (146-148). 4) Anergic CD8⁺ T cells with defective reactivity to antigen have been found repetitively in chronic HIV-infection (149-151). 5) Lack of perforin expression in HIV-specific CD8⁺ T cells and thus poor cytotoxicity has also been implicated in HIV-pathogenesis (152). 6) In late stage infection the release of IFN γ

by CD8⁺ T cells was also affected (153, 154). 7) Inefficient homing of HIV-specific CD8⁺ T cells to lymphnodes (LN) has been postulated to cause poor viral control of an important viral reservoir in LN, since the phenotypic analyses of HIV-specific tetramer positive cells revealed the absence of the LN-homing markers CD62L and CCR7 (141, 155). 8) CD8⁺ T cells from HIV-infected patients showed defects in TCR mediated signalling and in expression of the costimulatory molecule CD28 (156-158). 9) 'Skewed' or incomplete maturation of HIV-specific memory T cells devoid of important effector functions has also been correlated with poor viral control (159, 160).

However, this list is by no means complete and the relative importance of each of these (and other) mechanisms to explain the failure of CD8⁺ T cell mediated immune control of HIV-infection is largely unclear but most likely multifactorial. It remains very difficult to differentiate in a natural human infection between important pathogenic factors and epiphenomena. Ideally, established parameters of an infection, which are predictive of disease progression and outcome (i.e. VL and CD4-count in HIV-infection), are used to correlate with the measured variables. However, in immunologic studies of human viral infections certain key variables, which can crucially influence the outcome of a virus-host interaction, are not known or might be widely distributed between individuals of a 'experimental group'. These include parameters of the virus itself (infectious dose, pathogenicity, cellular tropism, virulence and subtype or strain), circumstances of the infection (exact time point and route of infection) and, in addition, genetic and other host factors.

2.3.3.2. T cell responses in LCMV-infection

Some of these variables can be controlled in mouse models of persistent infection. These models have clearly shown, that the basic parameters mentioned above have a crucial influence on the outcome of a virus-host interaction (161, 162). LCMV, which is one of the most widely used viral models in mice, is a poorly cytopathic arenavirus and the mouse is its natural host (163). It replicates widely in many different cell types and organs, but the infection is usually cleared in immunocompetent mice below the limit of detection (< 50 plaque forming units (PFU) / ml blood) within 10-20 days by a vigorous and perforin dependent CTL-response (164). Nevertheless, virus does persist at very low levels despite a high frequency of $CD8^+$ memory T cells (120, 165). The development of a neutralizing antibody response seems to be necessary to prevent re-emergence of the virus late after infection (166, 167). Therefore, initial virus control is CTL-mediated, whereas long term control probably requires the coordinate action of all arms of the adaptive immune system.

However, infection of mice with a high dose of virulent (fast replicating) virus given systemically (i.v.), leads to high level virus persistence ($> 10^5$ PFU / ml blood) by exhaustion of certain epitope specific $CD8^+$ T cells and anergy of others (146, 168, 169). Thus, these simple parameters (virus strain, dose and route of infection) determine the outcome of LCMV-infection - clearance of virus to very low levels with strong CTL-memory - or high-level virus persistence with deletion and/or anergy of specific CTL. Like this, LCMV has been used as a model for low level or high-level virus persistence. Furthermore, the exhaustive induction of virus specific $CD8^+$ T cells is greatly facilitated by a lack of specific $CD4^+$ T cell help (170, 171). It was speculated for a long time, that maintenance of functional LCMV-specific CTL depends on the presence of $CD4^+$ T cells (172, 173). However, a recent analysis revealed that neutralizing antibodies and not T

helper cells provided the necessary additional factor needed for long term control of LCMV (167). This is surprising in the light of the fact that CD4⁺ T cells were shown to be crucial for CD8⁺ T cells during the initial phase of antigen contact. Without T cell help in the primary response, CD8⁺ T cells failed to expand upon a second exposure to the same antigen (174-177). Since persistent infections are characterized by continuous exposure to antigen with the need for permanent 'secondary' responses, it would be expected that CD4⁺ T cell-deficiency leads to a failure of immune control in persistent infections.

Only in high level LCMV-persistence with viral titres remaining constantly in the range of 10⁴-10⁶ PFU/ml blood, deletion or dysfunction of specific CD8⁺ T cells was observed (168, 169). In low level persistence (< 50PFU/ml blood) LCMV-specific memory CD8⁺ T cells have excellent effector function and protective capacity unless the neutralizing Ab-response is prevented (166, 167).

2.3.4. T cell responses against viruses that persist at low levels

Herpesviruses are the prototype of viruses that persist at low levels. Since they are cytopathic, they have evolved the strategy of latency to survive in a host without causing permanent damage, and the strategy of reactivation to enable transmission to a new host. They have a large and rather stable genome of dsDNA, they replicate slowly and they encode between 100-200 different proteins, which provide many targets for the host's immune system. Nevertheless, they are evolutionary very successful, since most individuals harbour more than one herpesvirus, and they persist indefinitely in their respective hosts. During primary infection and reactivation they are visible for the host's immune system but not during latency (or only to a very limited extent). Their strategy of

immune evasion is one of 'camouflage' and 'sabotage' enabling them to persist despite a vigorous immune response (129).

2.3.4.1. T cells in EBV-infection

EBV is a γ -herpesvirus that mainly infects B cells via the complement receptor CD21 (178). Primary infection is usually acquired in childhood by transmission of virus in saliva. Interestingly, the age at the time of infection has an influence on clinical symptoms. The syndrome of infectious mononucleosis usually occurs in older children and young adults and only rarely in children infected at young age, most probably because of a less intense T cell response (179). Tetramer based analysis of the EBV-specific CD8⁺ T cell-response in infectious mononucleosis disclosed extremely high frequencies of EBV-specific T cells, which are mostly directed against proteins expressed during the lytic cycle of infection (122, 127). After resolution of primary infection and termination of the lytic cycle these responses are culled substantially and asymmetrically, which is not in agreement with the burst size model mentioned earlier (180). Sometimes they seem to be lost completely which is surprising, since viral reactivation with reexpression of lytic proteins frequently occurs to allow transmission to a new host. However, a different evolution has been described for CD8⁺ T cells specific for latent epitopes of EBV (127, 181). These responses develop more slowly and are mostly subdominant during acute infection. Later on they become the dominant responses in asymptomatic virus carriers. Although the virus has evolved a strategy to protect the most abundantly expressed latent protein EBNA-1 from CD8⁺ T cell attack by preventing its degradation by the proteasome (182), the EBV specific T cell response seems still to be important for the host also during latent infection. EBV-associated B

cell lymphomas are much more frequent in people which are immunosuppressed due to HIV-infection or after transplantation. In contrast to HIV-infection, there is no indication that EBV-specific CD8⁺ T cells are dysfunctional at any stage of the infection in otherwise immunocompetent individuals (127).

2.3.4.2. T cells after infection with murine γ -Herpesvirus-68 (MHV-68)

Infection of mice with the murine γ -Herpesvirus-68 (MHV-68) leads to a syndrome, which has similarities to EBV-infection in humans. The virus has a lytic and a latent replication cycle and persists mainly in B cells and macrophages. Late after infection about 10% of infected animals develop lymphoproliferative disease originating from B cells (183). The CD8⁺ T cell response is crucial for the initial termination of productive infection in the lungs (184). However, mice deficient for CD4⁺ T cells cannot maintain protective immunity and the virus reactivates mainly in the lung, leading to death after 100-120 days despite a prominent CD8⁺ T cell response which seems to be fully functional (185, 186). Again, the T help dependent neutralizing Ab-response seems to be more crucial than a direct antiviral effect of CD4⁺ T cells (183). However, analysis of B cell deficient mice is complicated by the fact, that B cells are a principal source of latent MHV-68.

Careful experiments comparing the CD8⁺ T cell response against a transient (i.e. influenza A virus) and a persistent respiratory infection (MHV-68) revealed no important differences during the acute phase of the response. During the memory phase the frequency, certain markers of activation and the rate of turnover of CD8⁺ T cells remained higher in persistent infection than after complete resolution of transient infection (126).

Overall, these studies of T cells in persistent viral infections suggest that high level persistence of a virus has profound and usually detrimental consequences for antiviral CD8⁺ T cells leading to deletion, exhaustion and/or anergy. This negative outcome seems to be enhanced by a lack of specific CD4⁺ T cell help or by a lack of neutralizing Ab.

Persistence of viruses at very low levels or their intermittent reactivation leaves the virus specific CD8⁺ T cell response phenotypically and functionally intact. There are increases in T cell frequency, activation level and rate of turnover without evidence for specific defects. However, lack of CD4⁺ T cells can also lead to loss of CD8⁺ T cell-mediated viral control in low level persistent infections (166, 185, 186). However, it remains to be analysed whether subtle functional defects of CD8⁺ T cells precede the re-emergence of viral progeny.

In our study we have analysed the course of the CD8⁺ T cell response during MCMV-infection of immunocompetent mice using modern immunological techniques. Our results, gained in a well-defined model of low-level persistent viral infection, contribute to the increasing knowledge about the interplay between viruses and their natural hosts. A unique pattern of a virus specific immune response emerged from this analysis and we propose an explanation for these findings, which is mainly based on the particular biology of CMV. We have performed additional experiments with recombinant MCMV, which suggest a possible way to harness the biology of these viruses for vaccine purposes. Finally, we try to put our results into a broader immunologic context of our current understanding how protective immunity is best maintained.

3. Material and Methods

3.1. Mice and animal experiments

Mice were purchased from Biomedical Services Unit, John Radcliffe Hospital, Oxford, UK. Animals were bred under specific pathogen free conditions and housed in a controlled conventional unit during the experiments. All animal experiments were performed with age-matched female BALB/c and C57BL/6 mice with permission of the home office according to the Animals (Scientific Procedures) Act 1986 (UK), requiring the use of minimal numbers of animals. Mice were infected with a volume of 200 μ l virus in PBS i.v. (MCMV, LCMV and VV) or i.p. (VV), or with 20 μ l virus in PBS intranasally (influenza). The infectious dose is indicated in the text or figure legend. A short (< 1min) anaesthesia with ether was applied for intranasal influenza infection. For s.c. immunization with peptide, 200 μ g of peptide was solubilized in 100 μ l PBS and then emulsified in 100 μ l of CFA. For flow cytometry, a 50 μ l blood-sample was taken from the tail vein.

Organs were prepared after intravenous perfusion with 15-20 ml PBS (4°C) immediately after sacrifice, followed by preparation of single cell suspensions by disruption of organs over a fine metal grid within 1hr. To separate lymphocytes, liver preparations were then centrifuged over Percoll (Sigma-Aldrich, Poole, UK) before staining. Other organ suspensions were centrifuged once to remove debris and directly used for staining and flow cytometric analysis. Broncho-alveolar lavage was performed by insertion of a fine canula into the trachea after perfusion, followed by direct lavage of PBS into the lungs. Cells were centrifuged and used directly for staining and flow cytometric analysis.

3.2. Viruses

MCMV (Smith strain, ATCC: VR-194) and Vac89 (187) were provided by Prof. U.H. Koszinowski, Max von Pettenkofer Institute, Department of Virology, Munich, Germany. MCMV was grown on mouse embryonic fibroblasts (MEF) and purified by sucrose gradient centrifugation according to established protocols (188). MCMV titres of virus stocks and organ homogenates were determined by virus plaque assays on MEF as described using centrifugal enhancement of infectivity (188). Titres are expressed as $\log_{10}(\text{PFU MCMV/organ})$. The detection limits of the assays are indicated in the figure legend. Influenza A virus H17 was provided by Dr Keith Gould, Imperial College, London. VacNP was provided by Prof. A. Townsend, Weatherall Institute of Molecular Medicine, Oxford, UK., and VacGP by Prof. B. Moss, Laboratory of Viral Diseases, National Institute of Health, Bethesda, MD (189, 190). Recombinant VV were grown and viral titres were measured by virus plaque assay on TK⁻ (thymidine kinase deficient) cells. Diluted and sonicated lysates of TK⁻ cells were used for infection. A single stock or the respective virus was used for the majority of experiments, importantly those following development of immunity over time.

3.2.1. Generation of recombinant MCMV

Recombinant MCMV were designed for the purpose of this project. They were generated according to a recently established protocol by Dr. Markus Wagner in the research lab of Professor Ulrich H. Koszinowski (Max Pettenkofer Institute in Munich, Germany (191)). Recombinant MCMV with a C-terminal fusion of the LCMV-derived GP33-41 or the Influenza-derived NP366-374 peptides (see below) to the non-essential ie2-gene of MCMV were generated using a new mutagenesis procedure for the MCMV genome

cloned as bacterial artificial chromosome (pSM3fr) in *E. coli* (192). It was shown that virus, reconstituted from the BAC genome pSM3fr, shows wild type-virus-like replication properties and cellular tropism in vitro and in vivo (193). For generation of the recombinant viruses MCMV-GP and MCMV-NP, two linear DNA fragments containing a kanamycin resistance gene, homologies of about 40 nts to the up- and downstream sequences of the stop codon of the *ie2* gene, and the GP33-41 or NP366-374 sequence, respectively, were generated by PCR. For generation of the fragment containing the GP33-42 sequence (and additional 6 flanking nts on each side) the contiguous primers *ie2*-LCMV-5 (5'-GAACCACGG GTTCTTTCT CTTGACCAG AGACCTGGT GACCGTCAG GAAGAAGAT TCAGGGTAT CAAGGCTGTC TACAATTTT GCCACCTGT GGGATATGA CGATTATTC AACAAAGCC ACG-3') (GP33-41 underlined) and *ie2*-Kn-3 (5'-CTGTCCGAT GAATAAAAC CTCTTTATT TATTGATTA AAAACCATG ACATACCTC GTGTCCTCG CCAGTGTGA CAACCAATT AACC-3'), and the template plasmid pACYC177 (NEB) were used for PCR. For generation of the fragment containing the NP366-374 sequence (and additional 6 flanking nts on each side) the contiguous primers *ie2*-Flu-5 (5'-GAACCACGG GTTCTTTCT CTTGACCAG AGACCTGGT GACCGTCAG GAAGAAGAT TCAGCAAAT TGCTTCAAAT GAAAACATG GATGCTATG GAATCATGA CGATTATTC CAACAAAGC CACG-3') (NP366-374 underlined) and *ie2*-Kn-3 (see above), and plasmid pACYC177 were used for PCR. The fragment was then transformed into DH10B for mutagenesis of the MCMV-BAC pSM3fr, which contains the whole MCMV genome (strain smith). Mutagenesis was performed following a recently published protocol that uses homologous recombination mediated by the recombination functions $\text{red}\alpha\beta$ from bacteriophage λ (194). Correct fusion of the

epitope sequences to the C-terminus of the *ie2* gene within the viral genomes was confirmed by restriction pattern analysis of the recombinant MCMV BAC with *NsiI* and by additional sequencing of the *ie2*-epitope fusion (data not shown). Expression of the IE2-epitope fusion was also confirmed by Western blot analysis (Fig 2A).

3.3. Peptides and tetramers

The following peptides defining MCMV-derived CD8⁺ T cell-epitopes in the MHC-haplotype H2^d from 5 different MCMV proteins were used: m123/pp89 (pp89, H2-L^d-restricted, ¹⁶⁸YPHFMPTNL¹⁷⁶ (37)), m04/gp34 (m04, H2-D^d-restricted, ²⁴³YGPSLYRRF²⁵¹ (195)), M84/p65 (M84, H2-K^d-restricted, ²⁹⁷AYAGLFTPL³⁰⁵ (26)), M83/pp105 (M83, H2-L^d-restricted, ⁷⁶¹YPSKEPFNF⁷⁶⁹ (27)) and m164 (m164, H2-D^d-restricted, ¹⁶⁸AGPPRYSRI²⁶⁵ (196)). As a negative control, a peptide derived from an endogenous retrovirus of the murine CT26 colon carcinoma cell line was used (CTpep, H2-L^d-restricted, SPSYVYHQF) (197). The following peptides defining LCMV and influenza derived CTL-epitopes were used: LCMV-GP (GP33-41, KAVYNFATC, H2-D^b-restricted (198) or GP34-41, AVYNFATC, H2-K^b-restricted (199)) and influenza-NP (NP366-374, ASNEMNDAM, H2-D^b-restricted (189)). Peptides were synthesized at a purity of >70% (Research Genetics, Huntsville, AL), diluted and used at the indicated concentrations.

The tetramers used in this study were produced as described (114). Briefly, recombinant H2-L^d, H2-D^b and H2-K^b-heavy chain were expressed in *E. coli* (BL21), purified from inclusion bodies, biotinylated enzymatically and refolded with human β 2-microglobulin and the appropriate peptide. Plasmids encoding for the different MHC class I heavy chains were generously provided by Awen Gallimore (Oxford University, UK) and John

D. Altman (Stanford University, CA). Refolded complexes were purified by HPLC and tetramerized using PE-labelled extravidin (Sigma-Aldrich, Poole, UK) at a molar ratio of 4:1. Specificity and sensitivity of tetramers was regularly tested on CTL-lines restimulated with the appropriate peptide. The control CT-tetramer was generously provided by Dr. A. Gallimore, John Radcliffe Hospital, Oxford, UK. In all experiments background staining with CT-tetramer was $< 0.1\%$ of CD8⁺ T cells (not shown).

Fifty μ l of peripheral blood or 2×10^5 - 10^6 nucleated cells from spleen, liver, lymph node, salivary gland and lung broncho-alveolar lavage (BAL) were prepared in cold PBS containing 2% FCS, 0.2% NaN₃ and 10 mM EDTA. Cells were stained for 20 min at 37°C with pp89- or CT-tetramer. Surface markers were quantified by staining for 30 min at 4°C with the following Ab (purchased from BD, San Jose, CA.): APC- or PerCP-labeled rat anti-mouse CD8 (clone 53-6.7), PerCP- or FITC-labeled rat anti-mouse CD45R/B220 (clone RA3-6B2), FITC-labelled rat anti-mouse CD18 (clone C71/16), FITC-labelled rat anti-mouse CD25 (clone 7D4), FITC-labelled rat anti-mouse CD43^{1B11} (clone 1B11), FITC-labelled rat anti-mouse CD44 (PgP-1, clone IM7), APC- or FITC-labelled rat anti-mouse CD62L (MEL-14), and a panel of FITC-labelled antibodies specific for the variable region of TCR β -chains (V β). FITC-labelled hamster anti-mouse CD69 (clone H1.2F3) was purchased from Serotec Ltd. Oxford, UK. FITC-labelled rat IgG2a (BD) and hamster IgG (Serotec) isotype controls were used for gate settings. Analysis was performed as described above.

3.4. Assessment of IFN- γ -secreting peptide-specific CD8⁺ T lymphocyte frequencies

Frequencies of IFN γ -producing peptide specific T cells in the spleen were quantified by intracellular IFN γ staining after stimulation with peptide (ICS) according to the protocols

of the supplier of reagents (Becton Dickinson, San Jose, CA. (BD)). After 6h peptide (10^{-6} M) stimulation, spleen cells were stained with allophycocyanin or PerCP[®]-labelled rat anti-mouse CD8 (clone 53-6.7), fixed and permeabilized and then stained with FITC-labelled rat anti-mouse IFN γ (clone XMG1.2) or with an FITC-labelled rat IgG1-isotype control Ab. Samples were analysed with a FACSCalibur[®] (BD) using Cell Quest[®] software. Gates were set according to the isotype control and frequencies are given as percentage of IFN γ -secreting cells of total CD8⁺ T cells. Background staining for IFN γ was below 0.05% of CD8⁺ T cells after stimulation with irrelevant peptides and after stimulation of cells from naïve mice.

Within the region of LCMV-GP, that is shared between MCMV-GP, VacGP and the peptide GP33-41 (³³KAVYNFATC⁴¹) used for immunization, two differentially restricted CD8⁺ T cell-epitopes exist in the H2^b-haplotype: H2-D^b-restricted GP33-41 and H2-K^b-restricted GP34-41 (198, 199). After in vitro stimulation with KAVYNFATC, specific CD8⁺ T cells with either MHC-restriction produce IFN γ , thus these percentages are the sum of GP33 and GP34-specific cells.

3.5. Assessment of MCMV-specific cytotoxic activity in vitro

CTL-activity of spleen cells was determined by ⁵¹Cr-release assay as described (190). Effector spleen cells were restimulated for 5 days in vitro with peptide pulsed (10^{-6} M) and γ -irradiated (25 Gy) spleen cells. Serial dilutions of bulk cultures were incubated for 5h with ⁵¹Cr-labelled and peptide pulsed (10^{-6} M) EL4 target cells (H2^b) and the supernatant was assayed for released ⁵¹Cr. Spontaneous ⁵¹Cr-release and unspecific lysis of target cells pulsed with irrelevant peptides was below 15% in all assays.

3.6. Bromo-deoxy-uridine (BrDU) labelling and calculation of cycling cell populations

Mice were treated with 0.8 mg/ml BrdU in the drinking water and injected daily with 1 mg of BrdU i.p. for 10 days. After 10 days the spleens were removed and tetramer staining was performed as above. BrDU staining was performed after permeabilisation of cells using a BrDU labelling kit according to manufacturer's instructions (BD, San Jose, CA).

3.7. In vivo protection assay

The ovary protection assay was performed as described (200). Briefly, mice previously infected or immunized as indicated in the text were challenged with 5×10^6 PFU recombinant VV i.p. Four days later, ovaries were homogenized and VV-titre was measured by virus plaque assay on TK⁻cells. Titres are expressed as $\log_{10}(\text{PFU VV/organ})$. The detection limit was 40 PFU VV/ovary in all assays. The level of protection is given as the percentage of virus titre reduction in the respective experimental group compared to age-matched naïve mice or mice infected or immunized with an unrelated virus/Ag.

3.8. Calculation of correlation between the frequencies of epitope-specific CD8⁺ T cells, the level of protection and the time after priming

Mice were primed with the indicated viruses and the frequency of epitope-specific CD8⁺ T cells was measured in the blood 0-3 days before challenge with recombinant VV. Four days after challenge VV-titres were determined in the ovaries of immunized and naïve age-matched mice. The level of protection was calculated by subtracting the $\log_{10}\text{PFU}$

VV-titre of the immunized mice from the mean \log_{10} PFU VV-titre of the naïve group of mice. The frequency of epitope-specific CD8⁺ T cells before challenge was then correlated with the level of protection and the time since priming. In addition, the level of protection was correlated with the time since priming. Correlations were calculated using Spearman's rank correlation using a logarithmic scale. The number of animals included into the calculations is indicated in the table.

4. Results

4.1. Chapter 1

Memory inflation: continuous accumulation of antiviral CD8⁺ T cells over time.

4.1.1. Summary

CD8⁺ T lymphocytes play an important role in the control of intracellular pathogens, both during acute and persistent infections. This is particularly true in the case of persistent herpesviruses such as human cytomegalovirus (HCMV), which are typified by large virus-specific CD8⁺ T cell populations during viral latency. To understand the origin of these populations and the factors shaping them over time, we investigated the CD8⁺ T cell-response after murine cytomegalovirus (MCMV) infection. The kinetics of the acute response was characterized by rapid expansion of activated T cells, followed by a contraction phase. Thereafter, we observed a striking pattern, where MCMV-specific memory CD8⁺ T cells steadily accumulated over time, with 20% of all CD8⁺ T cells at one year specific for one MCMV epitope. Accumulation of MCMV-specific CD8⁺ T lymphocytes was seen in all organs tested and was associated with continuous activation of specific CD8⁺ T lymphocytes, primarily within lymph nodes. The pattern of accumulation was observed only in two out of five epitopes tested, and was accompanied by a gradual restriction in T cell receptor V β chain usage over time. This novel pattern of a virus-specific CD8⁺ T cell-response suggests that continuous or repetitive exposure to antigen can slowly mould memory T cell populations over time. This may be relevant for understanding of the evolution of the large HCMV-specific CD8⁺ T cell populations seen in humans.

4.1.2. Introduction

To analyse the CD8⁺ T cell-response to another important persistent virus with contemporary methods, we have characterized the CD8⁺ T cell-response after infection with MCMV in detail using a combination of phenotypic and functional assays. We focused on the immunodominant CD8⁺ T cell-response directed against the IE1-protein m123/pp89 (pp89) after infection with MCMV and compared it to the same response generated after infection with a non persistent recombinant Vaccinia virus (VV) expressing pp89 (Vac89, (187)). We have also analysed responses directed against other recently described CD8⁺ T cell-epitopes, which are not derived from IE-1. The results presented here suggest that apparently latent MCMV has a continuous and profound influence on the frequency, phenotype and distribution of MCMV-specific CD8⁺ T cells and give an insight into the possible evolution of CMV-specific T cell responses in humans.

4.1.3. Results

4.1.3.1. Accumulation of functional pp89-specific CD8⁺ T cells after MCMV-infection

Initial experiments were performed to correlate the immune response against MCMV with the kinetics of viral production. The titre of replicating MCMV was determined by virus plaque assay (Fig. 1A for spleen and salivary gland; liver and lung not shown). In the spleen and liver the peak MCMV titre was reached 3 days after infection with titres falling below the limit of detection by day 12. In the lungs and salivary glands the peak was about a week later (day 10-15), and the decline slightly slower. By day 40, replicating virus was detectable only in salivary gland tissue in 33% of the infected mice. At all time points thereafter (day 75-400), productive MCMV-replication was below the detection limit for all organs tested. Thus, while the kinetics of viral growth and decline differed in different organs, after about 8-10 weeks replicating virus was no longer detectable.

We next measured the frequency of H-2L^d restricted, pp89-specific CD8⁺ T cells by tetramer staining (pp89-tet) or intracellular cytokine staining for IFN γ (pp89- IFN γ) after infection of BALB/c-mice with MCMV or Vac89. VV does not persist or reactivate after resolution of primary infection in immunocompetent mice (201) whereas MCMV establishes a latent infection in many sites with a continuous potential for reactivation (2). We thus compared the frequencies of CD8⁺ T cells directed against the same epitope following two different infections.

In the blood, an initial peak of 5.9% of pp89-specific was reached 8 days after MCMV-infection. A contraction phase then ensued and frequencies declined to 3.4% at day 15 and were maintained at this level until day 40 (3.7%). Remarkably, thereafter pp89-specific CD8⁺ T cell-frequencies began to rise again at a time when replicating MCMV

was no longer detectable. More than 20% of CD8⁺ T cells were specific for this single viral epitope 400 days after MCMV-infection (Fig. 1B).

In the spleen, a similar “biphasic” pattern was observed. Initial expansion of pp89-specific CD8⁺ T cells peaking at day 8-10 (5.9%) was followed by a contraction phase and from day 40 onwards a gradual increase in pp89-specific T cells. By day 400 15.3% of CD8⁺ T cells were specific for pp89 by tetramer staining and at least 80% of these cells produced IFN γ within 6h of antigen recognition (Fig. 1C). After an initial expansion and only limited contraction phase an accumulation of the total number of pp89-specific cells per spleen was also observed from day 40 to day 400 by 2-3-fold (Fig. 1D).

In contrast, after infection with Vac89, the peak of the pp89-specific CD8⁺ T cell-response was reached between day 6 and day 9 (2% of CD8⁺ T cells tetramer positive). By day 28 the frequencies had halved and were maintained at a constant level thereafter (about 0.7% of CD8⁺ T cells). Similar results were obtained using both tetramer and intracellular cytokine staining (Fig. 1B-E).

This accumulation of virus specific CD8⁺ T cells after MCMV infection was not restricted to the blood and spleen, as a similar pattern was observed in other organs. We analysed the frequency of pp89-specific CD8⁺ T cells in lymph nodes (cervical, mesenteric and axillary) and in organ infiltrates from liver, lung (BAL) and salivary gland over time with an increase in frequency during viral latency seen in all cases (Fig 2A). Additional data from other organs including ovaries, adrenal glands and bone marrow confirmed this general pattern (data not shown). Thus during latency, there is an accumulation in multiple body compartments of pp89-specific CD8⁺ T lymphocytes in

terms of both frequency amongst CD8⁺ T lymphocytes and absolute numbers (Table 1, Fig 1D).

4.1.3.2. Oligoclonality of pp89-specific CD8⁺ T lymphocyte population

We next addressed the issue of whether these expanding populations represented the accumulation of polyclonal CD8⁺ T lymphocyte populations, or oligoclonal populations with restricted TCR usage (as has been observed in HCMV (22, 202)). To study this directly *ex vivo*, we identified pp89-specific T cells using the tetramer and co-stained with a panel of V β specific TCR antibodies. We analysed the proportions of pp89-specific cells, which expressed TCRs of different V β -types over time. This experiment (Fig 2B) shows at later time points an increase in the proportion of the tetramer positive population, which use the V β 8.1 TCR chain. Early after MCMV infection (day 8-12), pp89-specific T cells used a diverse set of V β TCR chains. The V β 8.1/8.2 usage amongst the pp89-specific T cell population then increased over time: about half the tetramer positive population expressed V β 8.1 or 8.2 by day 270. As a negative control, V β -specific TCRs (e.g. V β 3), which are not expressed in the BALB/c mouse, were below 0.1% (data not shown), and the background expression of V β 8.1 in tetramer negative CD8⁺ T cells did not increase significantly over time. Thus we have shown a restriction of TCR V β chain usage amongst the tetramer positive population, which parallels the continuous accumulation of antigen-specific T cells during viral latency.

4.1.3.3. Phenotype of pp89-specific T cells early and late after infection with MCMV and Vac89

The phenotype of pp89-specific CD8⁺ T cells was analysed early and late after infection

with MCMV and Vac89. A panel of phenotypic markers was used to assess the activation and maturation state of pp89-specific CD8⁺ T cells. Ten days after MCMV-infection, pp89-specific T cells showed an activated phenotype in the spleen (mainly CD43^{1B11-hi} and CD62L^{lo}). Only 9% of pp89-specific CD8⁺ T cells were positive for the early activation marker CD69 and the percentage of CD25^{hi} CD8⁺ T cells was negligible (Fig. 3). The vast majority expressed the proinflammatory adhesion molecules CD44 and CD18.

Six days after infection with Vac89, specific CD8⁺ T cells showed slightly lower expression levels of CD43^{1B11} and CD69, while the expression level of CD44, CD18 and CD62L was comparable to those after MCMV-infection. During the memory phase MCMV- and Vac89-infection, pp89-specific CD8⁺ T cells were CD43^{1B11-lo}, CD44^{hi} and CD18^{hi}. However, there was a clear difference in the expression of CD62L: the majority of pp89-specific CD8⁺ T cells remained CD62L^{lo} late after MCMV-infection whereas after Vac89 infection, only a minority were CD62L^{lo}. In addition, as late as 400 days after MCMV-infection, a distinct population (11%) of pp89-specific cells expressed CD69 – slightly more than during acute infection.

We went on to analyse CD69 expression on tetramer positive CD8⁺ T cells in different organs. Strikingly, while moderate amounts of CD69^{hi} pp89-specific T cells could be seen in the spleen, blood, liver and lung, the frequency was significantly higher in the lymph nodes, often reaching 30% (Fig 4A). This pattern was reproducible across lymph nodes from the mesenteric, cervical and axillary groups. As controls, tetramer negative CD8⁺ T cells from lymph nodes in MCMV and Vac89 infected mice and naïve mice all showed much lower levels of CD69 expression (% CD69^{hi} < 10%, data not shown). The

relationship between the cells in the different compartments and the kinetics of activation are complex (203), but a reasonable conclusion from these experiments would be that antigen specific activation of pp89 specific CD8⁺ T cells is occurring within the LN throughout MCMV-latency.

4.1.3.4. Proliferation of pp89-specific memory CD8⁺ T cells late after MCMV-infection

To examine the proliferation of tetramer positive CD8⁺ T lymphocytes *in vivo*, we performed BrDU labelling experiments. After 10 days of labelling, the incorporation of BrDU into tetramer positive and tetramer negative CD8⁺ T lymphocytes was analysed in MCMV-infected mice. An example of such an experiment is shown in Fig. 4B. As expected, a very high proportion of tetramer positive cells have incorporated BrDU during the acute phase of infection (about 90%). The rate of incorporation of BrDU in tetramer positive cells during the latent or memory phase – 250 days later - was approximately one fifth of this. A small but consistent difference in the incorporation of BrDU into tetramer positive cells and total CD8⁺ T cells was seen (mean 16.7% v 12.7%), as has been noted in similar experiments after infection with murine gamma herpesvirus-68, where similar incorporation rates were obtained (204). The lower frequencies of pp89-specific cells late after Vac89 infection limit the information that can be obtained from such analyses during the memory phase. Overall therefore the rate of proliferation of tetramer positive cells as judged using this assay was sustained during MCMV-latency at a level substantially lower than that seen acutely but on a par with other persistent infections.

4.1.3.5. Long-term accumulation of memory cells is not uniform and is not restricted to

pp89-specific CD8⁺ T cells

To evaluate whether accumulation of MCMV-specific CD8⁺ T cells was restricted to the IE1/pp89-specific response, we measured specific CD8⁺ T cell frequencies for epitopes derived from 5 different viral proteins up to 200 days after MCMV-infection. Of these, only pp89 is generated during the IE-phase of MCMV-replication (37). gp34 (m04), p65 (M84) and probably the m164 gene product are expressed during the E-phase and the structural virion protein pp105 (M83) is expressed during the L-phase (26, 27, 195, 196). The MCMV-specific CD8⁺ T cell-response, as measured by intracellular cytokine staining for IFN γ was clearly dominated by pp89-specific and m164-specific cells during productive infection and particularly during latency. Ten days after infection, 3-4% of CD8⁺ T cells were specific for pp89 and m164 derived epitopes (Fig. 5A), while only 0.2-0.5% were specific for the other three epitopes (Fig. 5B). Thereafter, CD8⁺ T cell-responses for subdominant epitopes all declined to 0.07% (close to the limit of detection of the assay) and were maintained at such low levels without evidence for accumulation. This was in stark contrast to pp89- and m164-specific cells, which increased in frequency over the period of latency (Fig. 1, 5A&B; Table 1).

4.1.4. Discussion

CMV is a major pathogen, which infects most of the world's population. It causes little disease normally because it is well controlled by the host's immune system. A number of mechanisms are important but a key element is the CD8⁺ T lymphocyte response. However if these mechanisms fail, as a result of immunosuppression (e.g. during AIDS or after transplantation), the disease caused by CMV is very significant. Similarly, it is also a significant disease causing pathogen in the foetus and newborn. Understanding the immune response against CMV is of relevance for two main reasons: firstly because of its pathogenic potential and secondly because immune mechanisms are responsible for life long control of persistent CMV-infection. Emerging data suggest that in CMV-seropositive healthy individuals, a significant effort is expended daily in simply suppressing CMV reactivation (205, 206).

Of all the CD8⁺ T cell-responses against human virus infections uncovered with recently developed technologies, those against herpesviruses like EBV and CMV are by far the largest in frequency (115). After acute EBV-infection massive CD8⁺ T cell responses directed against lytic viral epitopes are culled substantially with resolution of lytic infection, while other responses become more prominent during latent infection (122, 127, 180, 207). Since acute HCMV-infection is usually clinically silent, very little is known about how antiviral CD8⁺ T cells evolve from primary infection into the large populations often seen in asymptomatic seropositive individuals (up to 10% of CD8⁺ T cells), particularly in the elderly (23, 87, 202, 208-210). This observation prompts the immediate question of how such responses emerge and what their evolution is in the long term. We have addressed these questions using the murine model of CMV-

infection. This allowed us to experimentally address the mechanisms behind the development of such populations and the factors behind their maintenance.

Most strikingly, after initial expansion and contraction phases the frequency of CD8⁺ T cells specific for some MCMV-derived epitopes continues to increase long after the resolution of primary infection. This accumulation – which we have termed memory “inflation”- was not uniform. CD8⁺ T cells specific for IE1/pp89 and for m164 clearly showed this phenomenon, but frequencies of cells specific for other viral epitopes did not increase. Enrichment of pp89- and m164-specific but not m04-, M83- and M84-specific CD62^{lo} effector memory CD8⁺ T cells in the latent phase of MCMV-infection has been recently observed and a role of these cells in control of viral latency was proposed (196, 211). In these studies, a model of MCMV-induced interstitial pneumonia after immunosuppression by irradiation with subsequent hematopoietic reconstitution with syngeneic bone marrow was used. By homeostatic proliferation of cells in the lymphopenic host, this procedure has the potential, to induce alterations of magnitude, function and phenotype of these CD8⁺ T cell-responses, which are not directly related to the virus-host interaction (212-214). However, our results confirm and extend these findings to a more natural situation of MCMV-infection of immunocompetent mice and provide a longitudinal analysis of the kinetics of virus specific CD8⁺ T cells.

Both epitopes, which showed an increase in population size over time, are already dominant in acute disease, but the pattern of CD8⁺ T cell accumulation is not restricted to IE-proteins nor to specific MHC class I molecules. From the experiments presented here we cannot conclude that immunodominance during early productive infection is necessary for later accumulation, since other factors within the virus-host balance might influence the long term kinetics of the CD8⁺ T cell response considerably. The initial

infectious dose used, although relatively high in this study, was not important in determining the later accumulation of cells, as similar results were obtained using infectious doses three orders of magnitude lower (Fig. 6A).

One reasonable explanation for the accumulation of IE1/pp89 specific CD8⁺ T cells would be, that this protein is expressed very early during the viral life cycle. Thus, expression of pp89 during viral reactivation events is more likely to occur than for proteins derived from genes with E- and L-expression kinetics. Indeed, mRNA for IE1/pp89 and IE2 has been found during MCMV-latency, but usually transcription does not proceed to those E- and L-genes studied to date, unless MCMV-reactivation is promoted by immunosuppression or allogeneic transplantation (8-11, 215). Thus, IE1 specific CD8⁺ T lymphocytes active in such a site might provide an early ‘checkpoint’ preventing progression of the virus to full reactivation. In addition, presentation of IE-derived CD8⁺ T cell-epitopes by macrophages, which are an important source of latent virus (216), is efficient despite MHC class I downregulation by MCMV, which strongly interferes with presentation in general of E- and L-derived CD8⁺ T cell-epitopes (38, 217).

M164 is not expressed under the same promoter as IE1/pp89, and its biological function is not yet clear. Although it is probably expressed during the E-phase, the epitope is efficiently presented despite MCMV-interference with MHC class I presentation (218). Whether this then provides a second ‘checkpoint’ is currently a matter of speculation. However, the crucial role of CD8⁺ T cells in the overall maintenance of MCMV-latency and prevention of reactivation has been clearly established, since combined depletion of critical immunological mediators (B cells, NK cells, CD8⁺ and CD4⁺ T cells), viral

recrudescence is extremely rapid (68). Accumulation of CMV-specific CD8⁺ T cells may therefore arise from the unique biology of CMV, where a large depot of virus exists in multiple sites, which is readily reactivated and which demands a rapid response from 'effector' memory T cells.

These patterns – inflationary responses to some epitopes but not others - may well be mirrored in HCMV, although there are important differences. Seropositive individuals often have persistently high frequencies of HCMV-specific CD8⁺ T cells with a narrow clonality suggesting antigen driven expansion (22, 202). These responses are readily detected against the HCMV-tegment protein UL83/pp65 – a response, which has no apparent parallel in MCMV. As shown in Fig. 5C, the positional and functional MCMV-homologues of UL83/pp65, namely M83/pp105 and M84/p65, elicit only weak CD8⁺ T cell responses without evidence for accumulation. More recently, strong CD8⁺ T cell-responses specific for HCMV IE1/UL123, the human equivalent of pp89, have been described suggesting a co-immunodominance for pp65 and IE1 (24, 25, 86, 210, 219). It has also been shown that MHC class I loading by UL83/pp65-derived peptides occurs efficiently by an alternative pathway (cross-presentation), before interference with antigen presentation by HCMV is fully operational (20). To our knowledge, no systematic study has described the long-term evolution of HCMV-specific CD8⁺ T lymphocyte responses, and their relative specificity, but our findings suggest that such a specific study would be of considerable interest.

Accumulation of pp89-specific CD8⁺ T cells was only observed when mice were infected with MCMV and not after infection with Vac89, indicating a crucial role of the

viral biology in driving these accumulating populations. Multiple patterns of 'memory' kinetics have been studied in murine systems, and to date these have roughly fallen into two patterns. Several viruses show large initial expansions followed by contractions, with a stable memory pool (the 'burst size model'), where the initial expansion of specific cells is followed by 90-95% contraction to stable memory T cell frequencies. Thus, the level of memory is determined by the peak of the acute response (120, 125). Vac89, which does not persist, may well follow such a pattern. On the other hand, for persistent infections this concept has already been adapted, since the frequency of memory CD8⁺ T cells stabilizes at higher levels than anticipated by the 'burst size model' (126). However, in none of the cases studied to date has this 'inflationary' pattern been observed, so this might represent yet another potential pattern for viruses where persistence is an important part of their biology.

The issue of different patterns is raised after infection with EBV, another persistent herpesvirus, where there seem to be at least two different sets of CD8⁺ T cell-responses (127, 180, 207). Frequencies of cells specific for lytic viral proteins usually showed the kinetic of a 'burst size response', with rapid but rather asymmetric contraction after the initial expansion. In contrast, the frequency of T cells specific for latent viral proteins was less dominant during acute infection and sometimes gradually increased, dominating the EBV-specific memory pool. Although some evidence is accumulating for programmed contraction of CD8⁺ T cell-pools after infection (220), in some situations where virus persists and where it represents a continuous threat to the host, this contraction may be less apparent for the total pool of epitope specific T cells. While some clones might be in contraction, others might re-expand due to re-encounter of

persisting antigen. Thus the exact viral biology becomes crucial in determining the shape of the immune response *in vivo*.

We have addressed additional questions about the mechanism behind this phenomenon. During primary infection with MCMV, pp89-specific T cells have an activated phenotype (Fig. 3), which is comparable to Vac89 infection. However, late after infection (day 100-400), there was a clear difference in CD62L-expression: > 80% of pp89-specific CD8⁺ T cells were CD62L^{lo} after persistent MCMV-infection but CD62L^{hi} after transient Vac89-infection. In mice, antigen-persistence has been correlated with long term maintenance of CD62L^{lo} 'effector' memory cells (221) and their preferential localization outside of lymphoid tissue (222). More recently protection against bacterial challenge with *Listeria monocytogenes* was shown to be mediated exclusively by CD62L^{lo} CD8⁺ T cells (223). However, *ex vivo* we did not find a significant functional difference in IFN γ production and cytotoxicity (not shown) between pp89-specific CD62L^{lo} effector memory cells after MCMV-infection and CD62L^{hi} 'central' memory cells after Vac89-infection. Nevertheless, the finding of accumulating 'effector' memory cells capable of patrolling solid organs like lung, liver and salivary gland supports the concept that latent virus is constantly initiating reactivation which is prevented by circulating MCMV-specific CD8⁺ 'effector' memory cells.

Consistent with this phenotype, we observed a striking activation of pp89-specific CD8⁺ T cells during viral latency. This was most marked within lymph nodes, where, as in other infectious model systems, the frequency of tetramer positive cells is rather low after iv infection compared to other secondary lymphoid organs (222). Remarkably, nearly one third of pp89-specific cells showed signs of recent activation one year after

infection (Fig. 4A). Prolonged maintenance of CD69^{hi} CD8⁺ T cells particularly within draining lymph nodes has also been observed after infection with Influenza A virus, which does not establish a persistent infection, although very late time points have not been similarly studied (203). It is likely that the localization of CD69^{hi} MCMV-specific cells is related to the distribution of peptide-loaded antigen presenting cells (APC), which may be concentrated in lymph nodes. In experiments with HCMV, cross presentation of IE1 derived epitopes by dendritic cells has been observed, so the localization of APC and virus infected cells may be distinct (224).

The fate of CMV-specific CD8⁺ T cells is not yet clear as some may divide and many may disperse into tissues thereafter or potentially die in situ. The data from the BrDU experiments gives some indication of the rate of proliferation of the pool, which appears marginally higher than that of tetramer negative cells. Although this assessment cannot account for redistribution of cells and death in tissues it does indicate that a difference exists between proliferation rates of the pp89 specific cells and other heterogeneous CD8⁺ T cells. Even if this difference is small it could account on its own for the slow accumulation seen after MCMV infection. A difference in proliferation rate of 0.03% per day, over a 200-day period, would account for a relative doubling in size of the tetramer positive population in comparison to the rest of the CD8⁺ T cell pool. Although a similar picture of proliferation amongst tetramer positive and total CD8⁺ T cells has been described after infection with persistent MHV-68, no such accumulation of tetramer positive cells has been documented (204). Further analysis of the rate and site of death of specific cells across the entire organism is therefore required to understand the gradual accumulation, which appears unique to this system. Also, even longer experiments will

be required to assess the long-term fate of these populations over the lifetime of the animal.

Recent reports on the phenotype of human CD8⁺ T cells after infection with several persistent viruses revealed a substantial heterogeneity dependent on the infecting agent, the time after infection and the particular epitope studied (127, 160, 225). In addition, they are also heterogeneous between individuals. The experiments shown here represent a controlled attempt to understand one particular outcome of a host-virus interaction but one which is still clearly relevant to man, as strongly emphasized by recent reports (226). The specific phenotype of CD8⁺ T cells is likely to be dependent on the individual and possibly unique biology of different viruses and their respective hosts. Nevertheless, the MCMV-specific CD8⁺ T cell responses described in this report followed unique kinetics during latency, which are unprecedented. These results are highly relevant to persistent human infections and present a potential new paradigm for understanding certain specific host-virus or host-vaccine relationships. Since infection with HCMV is widespread, understanding this phenomenon has potentially significant implications for the development over time of the 'normal' human immune system.

4.2. Chapter 2

Sustained expansion of CD8⁺ T cell responses driven by recombinant cytomegaloviruses

4.2.1. Summary

CD8⁺ T cells are critical for the control of many persistent viral infections such as HIV, HCV, EBV and cytomegalovirus (CMV). In most infections large CD8⁺ T cell populations are induced early but then contract and are maintained thereafter at lower levels. In contrast, CD8⁺ T cells specific for mouse (M) CMV have been recently shown to continue to accumulate after resolution of primary infection. This unique behaviour was restricted to certain epitopes, including an immunodominant epitope derived from the Immediate Early 1 (IE1) gene product. To explore the mechanism behind this further we measured CD8⁺ T cell-mediated immunity induced by recombinant MCMV expressing epitopes derived from influenza A virus or LCMV, placed under the control of an IE promoter. We observed that virus-specific CD8⁺ T cell populations were induced and that these expanded gradually over time. Importantly, these CD8⁺ T cells provided long-term protection against challenge without boosting. These results demonstrate a unique pattern of accumulating T cells, which provide increasing immune protection over time, independent of the initial immunodominance of the epitope, and indicate the potential of T cell-inducing vaccines based on persistent 'self-boosting' vectors.

4.2.2. Introduction

CD8⁺ T lymphocytes play a critical role in the control of persistent infections such as HIV, Hepatitis C virus (HCV), EBV and cytomegalovirus (CMV). Defining the nature of CD8⁺ T cells mediating immunological protection and the rules governing the maintenance of these cells is crucial not only for our understanding of the pathogenesis of such infections but also for the design of T cell-based vaccines. It is clear that memory T cell populations may persist in the absence of antigen or further triggering of their TCR (227-229). However, it is also clear that the biology of the inducing pathogen can strongly influence the magnitude and the quality of the resulting T cell populations. Recent studies using tetramers have revealed that human CD8⁺ T cell populations specific for different pathogens may show considerable heterogeneity in phenotype (127, 160). In murine models it has been shown that the recirculation pattern, the activation status and ultimately the protective capacity of memory T cells may also be influenced by the nature of the primary infection (223, 230-232). We have examined in detail the unique T cell responses induced by mouse (M) CMV-infection and explored the protective capacity of CD8⁺ T cells generated in response to these pathogens.

We evaluated this issue using recombinant MCMVs. These viruses express foreign epitopes derived from influenza A virus or lymphocytic choriomeningitis virus (LCMV) under an IE promoter. We tested the hypothesis that such viruses would prime epitope specific responses, which would expand during latency, regardless of their initial immunodominance, and regardless of the mouse strain used. Additionally we examined the protective capacity of these populations in challenge experiments. The data provide important insight into the biology of host-CMV interactions and provide a novel perspective in the search for T cell based vaccines.

4.2.3. Results

4.2.3.1. Accumulation of IE1/pp89-specific CD8⁺ T cells and association with protection against in vivo challenge:

To evaluate the accumulation of MCMV-induced CD8⁺ T cells and its relationship to the size of the initial response, BALB/c-mice were infected with low dose (10^3) or high dose (10^6 PFU) of MCMV i.v. We then measured the frequency of CD8⁺ T cells specific for a dominant H2L^d-restricted epitope derived from the immediate-early protein 1 (IE1/pp89) in blood and spleen by staining with tetramers and we characterised the phenotype and function of these cells longitudinally.

Previously, we have demonstrated that functional pp89-specific CD8⁺ T cells accumulate continuously after high dose MCMV-infection, reaching up to 20% of all CD8⁺ T cells ((233) and Fig. 6A). This accumulation of memory T cells over time is not dependent on a large initial CD8⁺ T cell expansion, since it was also evident after low dose MCMV-infection (Fig. 6A and Table 2). In this instance, a much lower initial peak of specific CD8⁺ T cells was generated, but subsequent accumulation during latency nevertheless was observed. Between 75-85% of these accumulating pp89-specific CD8⁺ T cells remained CD62L^{lo} after both high and low dose infection in blood and spleen, which is characteristic for effector memory cells. More than 90% of pp89-specific cells were immediately functional as assessed by ICS (data not shown). The kinetics of accumulation in the blood closely reflects the total number of cells within the spleen (Table 2) which is similar to our previous observation demonstrating accumulation of pp89-specific T cells also in liver, lung, salivary gland and lymph node (233).

To test the *in vivo* protective capacity of pp89-specific CD8⁺ T cells, mice were challenged at various time points after MCMV-infection with pp89-expressing Vac89 (usually 10⁷ PFU), and 4 days later, the VV-titre were determined in the ovaries. Challenge with recombinant VV has been widely used to test the level of T cell-mediated protective immunity *in vivo* (200, 231, 234-237). The stringency of the assay varies with the genetic background of the respective recombinant VV, with the dose of VV-challenge and with the time interval between challenge and analysis.

Mice were completely protected against Vac89-challenge from day 10 until day 272 after infection with a high dose of MCMV (10⁶ PFU, Fig. 6B). We observed no protection after challenge with an unrelated VV at any time point after priming, indicating that protection was pp89-specific and was not significantly influenced by natural or heterologous immunity (not shown). After infection with a low dose of 10³ PFU MCMV, no significant protection was observed until day 20. Protection then increased until day 75 (when VV-titre were 99% lower than in naïve animals) and this level of protection was maintained until day 177. Using a lower dose of VV for challenge (10⁶ PFU), 4/4 mice were completely protected against challenge on day 242 (Fig. 6B). Overall, for mice primed with 10³ PFU MCMV, the level of protection against challenge increased gradually over time after MCMV-infection and thus correlated with increasing frequencies of pp89-specific T cells (Table 3).

Protective capacity was associated with a marked expansion of pp89-specific CD8⁺ T cells *in vivo* after VV infection. These expansions were antigen-specific, they occurred in spleen (Fig. 6C), blood, liver, lymph node and ovaries (data not shown) and they showed a positive correlation with the *in vivo* protective capacity. Interestingly, the

magnitude of the expansion seemed to correlate with the time after MCMV-infection (Fig. 6C).

4.2.3.2. *CD8⁺ T cell-induction by recombinant MCMV*

To test the potential of MCMV to induce CD8⁺ T cell responses against recombinant IE-expressed epitopes and to evaluate whether memory inflation would also occur in a different genetic background of the host, we generated two different recombinant viruses encoding two H2-D^b-restricted murine CD8⁺ T cell-epitopes. They were derived from Influenza A virus nucleoprotein (NP366-374, MCMV-NP) and from lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP33-41, MCMV-GP) and included two additional N- and C-terminal flanking amino acids. Recombinant epitopes were introduced into MCMV-IE2, which is dispensable for in vitro and in vivo viral replication, and is expressed before MHC class I interference by MCMV becomes operational during the early (E) phase of viral replication (2, 38). In addition, IE2-transcripts have been found during MCMV-latency (10).

Mouse embryonic fibroblasts (MEF) infected with MCMV-NP and MCMV-GP expressed IE2 similar to wild type (wt) virus, when transcription and translation of viral IE-proteins were synchronised with metabolic inhibitors. As expected, the IE2-protein of the recombinant viruses was slightly larger than of MCMV-wt due to the inserts (Fig. 7A). Replication kinetics and cell- and tissue tropism of recombinant viruses was comparable to MCMV-wt in vitro and in vivo (not shown).

Ten days after infection of C57BL/6-mice with 2×10^6 PFU MCMV-NP or MCMV-GP, ex vivo cytotoxic activity was not detectable by ⁵¹Cr-release assay (not shown) and tetramer staining was at or below the limit of detection ($\leq 0.15\%$ tetramer positive cells

of CD8⁺ T cells; Fig. 7C, ex vivo). After 5-7 days of in vitro restimulation, cytotoxicity specific for recombinant epitopes and staining with the respective tetramer were strongly positive (Fig. 7B and 7C, restim.). These results demonstrate that priming of CD8⁺ T cells specific for recombinant epitopes expressed in MCMV-IE2 did occur early after infection, but the responses induced were initially of low magnitude.

4.2.3.3. Slow accumulation of NP-specific CD8⁺ T cells after infection with MCMV-NP and correlation with protective immunity

We next analysed NP-specific CD8⁺ T cells emerging over time after infection with influenza or MCMV-NP (238). We used several phenotypic markers to analyse activation status and presumable recirculation pattern of NP-specific CD8⁺ T cells. In particular CD62L-expression was used to differentiate between 'central' memory CD8⁺ T cells (CD62L^{hi}) and effector memory cells (CD62L^{lo}) (221, 223, 233, 239). We compared the responses generated after persistent infection with recombinant MCMV with those elicited by the same epitope after natural, acute and transient influenza infection.

After influenza infection, NP-specific CD8⁺ T cells rapidly expanded reaching a peak of 4-5% of CD8⁺ T cells after 8-10 days in the blood (Fig. 8A and 9A) and a total number of $5.6 \pm 2.8 \times 10^5$ cells per spleen (Table 2). More than 95% of the cells were CD18 and CD44-positive but CD62L^{lo} and 75-85% produced IFN γ ex vivo (Fig. 8B, ICS, CD18 and CD44 not shown). Early after intranasal influenza infection NP-specific cells in the spleen were mostly low in expression of CD69 but high in expression of CD43^{1B11} (not shown) which is compatible with recent activation (203, 240).

Thereafter, NP-specific CD8⁺ T cell populations contracted by 90-95% and were maintained at a stable memory level of 0.5% of CD8⁺ T cells in the blood and $3.9\text{-}5.2 \pm 2.2 \times 10^4$ NP-specific cells per spleen (Table 2). As demonstrated previously, within a year after influenza infection a substantial proportion of NP-specific CD8⁺ T cells (40-60%) regained expression of CD62L, typical for resting or central memory cells (Fig. 8C for day 220; (241)).

After infection with recombinant MCMV-NP a completely different pattern of CD8⁺ T cell-expansion emerged. Up until day 20, the frequency of NP-specific CD8⁺ T cells was generally below or just at the limit of detection by tetramer or ICS reaching $2.5\text{-}2.9 \pm 1.6 \times 10^4$ NP-specific cells per spleen (Table 1, Fig. 8A & 9A, ICS not shown). If NP-specific cells were measurable by tetramer staining early after MCMV-infection (and not below the limit of detection), they expressed high levels of CD18 and CD44 but they were consistently low in CD69, CD43^{IB11} and CD62L suggesting previous contact with antigen but a lower level of activation than after influenza infection (not shown and Fig. 8B).

Slowly but steadily, frequencies of NP-specific T cells increased over time, reaching a level of 1.5% of CD8⁺ T cells by day 120 ($12.7 \pm 4.8 \times 10^4$ cells/spleen). Thereafter, we observed no further increase but rather fluctuating T cell frequencies ranging between 0.7-1.8% ($9.15 \pm 5.3 \times 10^4$ cells/spleen). In the memory phase, 85-90% of NP-specific CD8⁺ T cells maintained a CD62L^{lo} effector memory phenotype (Fig. 8B for day 220) and produced IFN γ ex vivo (ICS not shown).

To compare the level of protection generated after infection with influenza with that generated after MCMV-NP infection, mice were challenged at different time points with

5×10^6 PFU VacNP i.p. Four days later, we measured the VacNP-titre in the ovaries.

These three viruses only share one single CTL-epitope (NP366-374).

As expected from the frequency of functional NP-specific T cells, mice were highly protected against VacNP-challenge during the first 50 days after influenza infection (Fig. 9B, upper panel). Thereafter, protection was partially and gradually lost in parallel to the loss of NP-specific memory cells resulting, as expected, in a negative correlation between the time after influenza infection (priming) and the level of protection of $c = -0.736$ ($p < 0.001$, Table 3). However, on average, VacNP-titres were still 96% reduced compared to naïve mice 430 days after influenza infection.

In contrast, over the first 35 days, mice infected with MCMV-NP were not protected against VacNP-challenge. Only by day 50, when NP-specific $CD8^+$ T cells had accumulated sufficiently, a significant level of protection (99.97%) was reached, comparable to mice infected with Flu (Fig. 9B, lower panel). A high level of protection was maintained thereafter, correlating roughly with the frequency of NP-specific $CD8^+$ T cells ($c=0.507$, $p=0.014$), although there was considerable variation between individual mice late after priming. Nevertheless, the level of protection was positively correlated with the time since priming with MCMV-NP ($c=0.513$, $p=0.012$, Table 3).

4.2.3.4. Protective $CD8^+$ T cell-mediated immunity after infection with MCMV-GP

We used the same system to analyse immunity generated against a second antigen, in this case derived from LCMV. We compared the GP-specific $CD8^+$ T cell-response after infection with MCMV-GP, after infection with VV recombinant for LCMV-GP (VacGP) and after immunization with GP33-41 peptide emulsified in CFA (Fig. 10A). The

response generated by VacGP was maximal on day 6 and then dropped to a stable memory level of 0.4% of CD8⁺ T cells. After immunization with GP33 in CFA, GP-specific T cells expanded to 1.9% within 10 days and were then maintained at a nearly constant level for 120 days reflecting the granulomatous inflammation and delayed antigen release provoked by CFA. However, total numbers of GP-specific cells per spleen dropped from $25 \pm 9.7 \times 10^4$ on day 10 to $10.9 \pm 2.6 \times 10^4$ on day 120 (Table 2). In contrast, after infection with MCMV-GP, GP-specific CD8⁺ T cells also showed the pattern of 'memory inflation' as demonstrated previously for NP-specific T cells after infection with MCMV-NP. By day 120, about 1.3% of CD8⁺ T cells were GP-specific in the spleen. Overall, the total number of GP-specific cells per spleen increased from $1.1 \pm 0.5 \times 10^4$ on day 10 to $15.5 \pm 2.3 \times 10^4$ on day 120 (Table 2).

Mice infected previously with MCMV-GP or immunized with GP33+CFA were then challenged with VacGP, and VV-titre was measured in the ovaries. GP33+CFA induced partial protection from day 10 until day 65 of about 99% compared to naïve mice. By day 250 however, protection was lost completely (Fig. 10B, upper panel). In contrast, protection generated by infection with MCMV-GP developed more slowly within 40 days (99.7%), but was then maintained with considerable variation between individual mice (Fig. 10B, lower panel).

4.2.4. Discussion

In this study we have demonstrated that the pattern of continuously accumulating CD8⁺ T cell responses after CMV-infection is neither restricted to certain T cell epitopes nor to a particular strain of inbred mice. Accumulation was observed for immunodominant and for subdominant epitopes, it was independent of the dose of infection and even T cells specific for recombinant epitopes accumulate over time. These T cells are of the effector memory phenotype and provide – as they accumulate - long term protective immunity. These findings have important implications for our understanding of the immunological long-term control of CMV infection in mouse and, potentially, humans. In addition, they provide a first proof of principle for the use of persisting vectors to maintain or even increase protective CD8⁺ T cell responses without external antigen re-exposure.

Efficient induction of protective CD8⁺ T cell-responses by crosspriming with non-replicating antigens has proven to be a difficult task to achieve despite constant refinement of vectors and immunization protocols and despite recent improvements of our understanding how innate immune mechanisms direct and enhance the adaptive immune response (242). It is even more challenging to develop practical immunization regimes capable of maintaining T cell-mediated protection over prolonged periods of time, and it has been suggested that persistent antigen is required (231, 236). Our results support and extend these findings: CD8⁺ T cell-mediated protection against challenge with recombinant VV decreased late after infection with live influenza. Loss of protection correlated with the decreasing frequency of memory T cells and particularly within the subset of effector memory cells. This was despite a very potent primary CD8⁺ T cell-response, which was quantitatively at least 30-fold higher after influenza infection compared to infection with MCMV-NP (NP-specific CD8⁺ T cells/spleen on day 8:

7×10^5 vs. $< 2 \times 10^4$). Similarly, protective immunity was lost late after priming with synthetic GP33-peptide, despite the use of a very potent adjuvant, inducing inflammation and slow antigen-release (243). In addition, protection against challenge with LCMV is known to decrease within 80 days after priming with recombinant VV expressing LCMV-GP (231, 236)). These different priming conditions, although very efficient initially, share the feature of transient antigen delivery to $CD8^+$ T cells. Conversely, protective immunity increased over time after priming with recombinant MCMV. This correlated with accumulation of epitope-specific effector memory T cells.

The cells generated by MCMV infection have the characteristics of 'effector' memory T cells, possessing rapid effector function *ex vivo* and more importantly, providing protection *in vivo*. The phenotype shown is probably similar to the 'mature' memory phenotype associated with HCMV-specific T cell populations in man (87, 160, 210). These cells remain low in CD62L and may therefore effectively patrol and protect nonlymphoid organs. Indeed, in addition to accumulation in blood and spleen as demonstrated here, accumulation of memory T cells in other organs (liver, salivary gland, lung, ovary etc) has been recently demonstrated in BALB/c mice (233). Similarly, after challenge with recombinant VV, very striking expansion of virus specific $CD8^+$ T cells occur rapidly in the spleen (Fig. 6C) and in the liver (up to 40% of infiltrating $CD8^+$ T cells; not shown). Furthermore, these $CD8^+$ T cells were efficient to protect from VV replication in the ovaries. It has been demonstrated that the protective capacity of an anti-*Listeria monocytogenes* response induced by vaccination, is strongly linked to CD62L expression and thus to the recruitment and redistribution characteristics of $CD8^+$ T cells (223). Recent data has, however, suggested that in other situations the protective

capacity of central memory T cells generated from TCR-transgenic mice is even more significant compared to effector memory cells, and this has been linked to an increased proliferative capacity of central memory cells (237). Although the CD8⁺ T cell-populations after MCMV-infection (pp89-, NP- and GP-specific) were not pure, they are largely effector memory (>75%) and nevertheless did show strong protective capacity (Fig. 6B, 9B & 10B) and massive expansion upon challenge (Fig. 6C), suggesting that these experiments may depend on the exact nature of the priming and its duration, and the exact nature of the challenge.

Although formal experimental proof is lacking, a reasonable explanation for this unique pattern of accumulating CD8⁺ T cells, mediating increasing long-term protection, is persistent exposure to antigen. This is supported by recent virologic studies in MCMV: transcription of IE1 and IE2 (the gene loci, where the recombinant epitopes were inserted) occurs randomly during MCMV-latency and the frequency of these events seems to depend on the amount of latent MCMV-genomes (8, 10, 244). These are the first steps of any reactivation event of MCMV. Whether or not the reactivation proceeds further to transcription of early and late genes is likely to be under immune control and does usually not occur. CD8⁺ T cells have been shown to be most crucial to maintain MCMV-latency (68). Therefore, CD8⁺ T cells specific for IE1- and IE2-derived epitopes will encounter their cognate antigen repetitively by early reactivation events in MCMV-latency leading to endogenous boosting. Moreover, MCMV-dependent MHC class I downregulation is not yet operational during the IE-phase of viral reactivation attempts, contributing to efficient presentation of these epitopes (38). Interestingly, we and others have found similar accumulations of CD8⁺ T cells specific for an epitope derived from

m164, which is not expressed under the IE promoter (Fig. 5B and (196, 218)). It is possible that recombinant epitopes inserted in this and potentially other gene loci may show similar patterns of immunogenicity.

Clearly, we have not used specifically developed vaccine vectors, adjuvants or sophisticated immunisation regimes in our control groups, which would certainly improve the efficiency of CD8⁺ T cell-induction. Nevertheless, we have used the original pathogen (influenza) in a major part of this study and most modern vaccines are still less efficient to induce immune responses than the natural pathogens (245). However, we have not selected the recombinant MCMV for high expression of recombinant epitopes *in vivo*, for their optimal processing or for expression of additional immunostimulatory effects and we have used a similar straightforward immunisation protocol for recombinant MCMV. Additionally, our recombinant viruses still express the whole 'machinery' of immune deviation and subversion, particularly concerning MHC class I function (38, 246). It seems quite likely that deletion of some of these genes might lead to an attenuated viral phenotype while increasing immunogenicity, since the induction of an antiviral immune response becomes more potent without immune deviation. This has indeed been demonstrated recently for an MCMV deletion mutant of m152, an MCMV-E-gene responsible for retention of MHC class I peptide complexes in the cis-Golgi compartment (247). It will therefore be important to demonstrate whether attenuated MCMVs retain the characteristics of protective memory T cell accumulation described in this study.

In relation to man, recent studies have shown that very large numbers of functional HCMV-specific CD8⁺ T cells are present in seropositive individuals long after resolution of primary infection (23, 87, 202, 208, 210). In the elderly such responses comprise a significant proportion of the total lymphocyte pool (209, 248). In addition, certain activation markers such as CD69 are over-represented in HCMV-specific CD8⁺ T cells suggesting recent encounter of antigen (249). However, since primary infection is usually clinically silent, we know little about the longitudinal evolution of these massive HCMV-specific CD8⁺ T cell-pools over time. Clearly, further studies are needed to evaluate whether the principle of 'memory inflation' in mice also applies to certain HCMV-specific T cell responses in humans.

The most likely explanation for these massive HCMV-specific T cell populations in healthy seropositive individuals is, that HCMV like MCMV constantly initiates reactivation during clinically latent infection (8, 10). This leads to a repetitive (or possibly constant) triggering of HCMV-specific memory T cells, which both prevent reactivation and lead to accumulation of T cells over time. Whether memory inflation is needed to maintain virologic control or whether a stable level of memory T cells would perform the same function, remains speculative. Addressing this question requires very detailed analysis of the extremely low viral load of MCMV or HCMV and its distribution over time.

The pattern of CD8⁺ T cell responses demonstrated here is unique. To exploit this type of immune response for the development of a safe vaccine, a persistent CMV-based vector would have to be engineered to allow latent infection with regulated recombinant protein expression without the possibility of productive reactivation. Vaccines based on

life attenuated herpesviruses already exist in the form of those directed against Varicella Zoster virus (VZV, (250, 251)). There, the benefits of protection against disease and the risks of reactivation of VZV vaccine strain need to be carefully balanced (252-254). Whether we will be able to harness the biology of these viruses to generate safe, successful recombinant vaccines for complex infectious diseases like HIV, HCV, tuberculosis and malaria, as well as tumours such as melanoma is a challenge for the future (255). Additionally, it will be important to establish, in careful long-term experiments, whether any of the current DNA-based regimes possess the potential to recruit antiviral effector cells over extended periods of time. On the basis of the evidence presented here, we would argue, however, that for T cell based approaches, attenuated but persisting vectors such as recombinant herpesviruses should be seriously evaluated.

5. General discussion

5.1. Phenotype and function of T cells in persistent viral infections

Phenotypic analysis of virus- and tumour-specific CD8⁺ T cells has recently gained a lot of attention since the tetramer technology has facilitated ex vivo identification of specific T cells and their subsequent analysis by flow cytometry (115). If these phenotypic analyses are combined with functional data they can provide previously inaccessible information. Without functional ‘upgrade’, the interpretation of the relevance of a particular phenotypic profile is very difficult and prone to over-interpretation due to an inherently high variability of these biologic parameters, particularly if they are derived from small scale and crosssectional studies in humans. In addition, if tetramers are used to isolate CD8⁺ T cells for further in vitro analysis, it is important to keep in mind, that these reagents can activate CD8⁺ T cells via their TCR (256). Thus, the phenotype and the function of the cells may be altered during isolation compared to the in vivo situation.

Several concepts of the longitudinal differentiation of human memory T cells were derived mainly from phenotypic analyses of antigen specific T cells by FACS. Based on differences in the surface expression of the chemokine receptor CCR7 and the LN homing marker CD62L, Lanzavecchia and co-workers proposed to subdivide memory T cells into two main populations of ‘central’ and ‘effector’ memory cells. Within this framework central memory cells are positive for both CCR7 and CD62L, whereas effector memory cells are CCR7- and CD62L-negative (239). Initially, it was also postulated that the difference in surface CCR7- and CD62L-expression was linked to an important functional difference concerning immediate effector function, measured by ex

vivo cytokine production, which was restricted to effector memory T cells. This functional difference was not confirmed by other studies first in mice and later in humans (257, 258).

However, the differential expression of CCR7 and CD62L leads to a different recirculation pattern of central and effector memory T cells. Central memory cells primarily recirculate through and home to secondary lymphoid organs and only become activated by re-exposure to antigen, if the antigen is sufficiently drained into the lymphoid system. Upon activation central memory cells start to proliferate, differentiate into effector cells and migrate to the peripheral (non lymphoid) organs to eliminate the antigen. In contrast, effector memory cells inherently patrol through the peripheral sites of the body, where they were shown to exert immediate effector function upon antigen contact, particularly *ex vivo* cytotoxicity (222). Therefore, the lag phase between antigen contact in the periphery and effector function is potentially much shorter for effector memory cells. This has important implications, since immediate control of a peripheral infection (which is notable the most natural route of infection) would thus require effector memory cells. However, analyses of the protective capacity of effector and central memory cells have revealed conflicting results: older studies, which were performed before the concept of effector and central memory was established, suggested, that protection against peripheral viral challenge was crucially dependent on the presence of cells recently activated by antigen (effector or effector memory cells), whereas protection against systemic viral challenge was efficiently provided by resting (or central) memory cells (231, 236). In *Listeria monocytogenes* infection, CD8⁺ T cells with a phenotype of central memory cells primed with killed bacteria were inefficient to protect against a secondary challenge (223). In contrast, in a different experimental

setting using LCMV-specific transgenic T cells, protection against challenge infections mediated by adoptively transferred central memory cells was superior to effector memory cells. This protective effect was linked to the increased proliferative potential of central memory cells (237).

Our own experiments suggest that maintenance of substantial populations of effector memory T cells after priming with MCMV was clearly associated with protection against challenge with recombinant VV. Protection was lost preferentially in situations where mainly central memory cells were maintained (Fig. 9B & 10B, upper panels). In addition, we did not find evidence for a proliferative defect of effector memory cells (Fig. 6C). This is supported by a recent study of lung infiltrating T cells during respiratory Sendai virus (SV) infection (259). After adoptive transfer of similar numbers of SV-specific effector and central memory cells the former proliferated more rapidly than the latter and subsequent long-term memory cells were mainly derived from effector memory cells. Unfortunately, protection against disease provided by effector as opposed to central memory cells was not analysed in this study.

However, in our analysis we did not perform cell separations according to CD62L-expression followed by adoptive transfer but we challenged the primed hosts directly. Since effector memory and central memory cells coexist at different ratios within the primed host, their respective contribution to protection was not assessed separately. Therefore, our evidence in favour of a crucial protective role of effector memory cells is more indirect than the experiments performed by Wherry et al. who suggest the opposite (237).

These studies might have important influences on the potential strategies to induce protective T cell memory and on future vaccine design. Based on these results it was

recently suggested to optimise vaccines according to their ability to induce and maintain central memory T cells (260). We think that this conclusion might be premature, since the data are derived from a single experimental system mainly using transgenic T cells. In addition, as pointed out above, conflicting data on the subject already exist in the literature and it is possible, that the outcome of these studies is crucially influenced by details of the experimental design including the nature of antigens and adjuvans used, the exact timing, dose and route of priming and challenge and the genetic background of the animals. The transfer of these data to human vaccine studies is yet a completely different issue.

Therefore, further experiments are clearly needed to firmly establish 1) whether the model subdividing memory T cells in central and effector memory cells is truly a general principle of T cell biology in mice and in humans, 2) whether and how the model applies to transient and chronic infections, 3) whether the proliferative and particularly the protective capacity of central and effector memory cells are different and 4) what signals are needed to preferentially induce and maintain central or effector memory cells.

The last two points might have highly relevant implications for future vaccine design, particularly if the presence of one of these cell populations would reflect a correlate of protection in an important human disease.

5.2. Protective CD8⁺ T cell mediated immunity: efficient priming versus continuous restimulation

Since many years immunologists debate about how immunological T cell memory is best maintained. During the last century the general conception was that memory was largely antigen independent. The famous measles outbreak on the remote Faroe islands,

where protective immunity survived during 65 years between epidemics without evidence for sporadic cases, was often cited as a striking example for antigen independence of memory (227). However, since measles virus is able to persist in a host for prolonged periods of time, this might actually be an example for antigen dependence (261, 262). Studies during the early 90ies then suggested that maintenance of T cell memory is dependent on persisting antigen (263, 264). This view was again challenged first by the demonstration of long term survival of memory cells after adoptive transfer into antigen free recipients (265) and later by experiments demonstrating long term maintenance of memory CD8⁺ and CD4⁺ T cells in an environment deficient of the restricting alleles of MHC class I or class II, respectively, excluding restimulation of T cells via engagement of their TCR by persisting antigen (228, 229, 266).

These studies have convincingly shown that memory T cells can be maintained without the need for persistent antigen. However, the debate has shifted to the question whether maintenance of memory T cells is equivalent to maintaining protective immunity (227, 267). Some of this controversy is related to the definition of memory: operationally, immunological memory is defined by an enhanced reactivity of the immune system to a pathogen or antigen that has been previously encountered. This usually reflects the pre-existence of a clonally expanded population of antigen-specific lymphocytes (268). Some would argue that an ongoing low level immune response to a persisting antigen would not fall into this definition of memory, since this is not a re-encounter but a persistent encounter of antigen (227). Therefore, persistent effector cells and not memory cells are responsible for the enhanced immune reactivity. For persistent infections, we might use the term infection/immunity, infectious immunity or concomitant immunity, as it was suggested by Mackaness more than 30 years ago (269). However, does any

level of antigen persistence beyond a certain time point after infection disqualify for 'true' memory? What would be the appropriate time point for complete antigen elimination? In such a purist concept of memory, adoptive transfer situations only would qualify for memory after infection with replication competent organisms, since antigen persistence at remote sites of the host or at very low levels is difficult to exclude: something we have learned from measles virus infection in man and LCMV-infection in mice (165, 261, 262). Clearly, such a narrow definition of memory excluding the possibility for antigen persistence does not reflect the natural interactions between pathogen and host and has not been selected for during evolution of the immune system. Therefore, rules for optimal memory cell persistence developed in experimental systems which exclude persistent antigen as a confounding factor should not form the basis for vaccine design (270). Nevertheless, these experiments have greatly enhanced our understanding of the minimal requirements for memory T cell survival.

Concerning antigen requirements for the maintenance of protective immunity, conflicting results have been generated dependent on the exact experimental system and the readouts for protection which were used (227, 228, 231, 236, 267, 271). Although the research groups of R. Zinkernagel and R. Ahmed both work mainly with the model of LCMV-infection to analyse T cell memory, published results seem to support opposing concepts: the former maintain the case for a crucial role of persisting antigen while the latter provide data for antigen independence of protective CD8⁺ T cell mediated immunity (267, 272). Some of their discrepancies are probably related to subtle differences in the experimental design of challenge experiments. The antigens or pathogens used for challenge (cytopathogenicity, cell and tissue tropism, replication capacity, virulence), the exact route and dose of their administration and the interval

between challenge infection and tissue sampling may all have a crucial influence on the outcome of a protection experiment. In general, the level of previously existing protective immunity needs to be higher, both quantitatively in terms of precursor frequency and qualitatively in terms of pre-activation status of T cells, 1st for pathogens that replicate in the periphery and not within secondary lymphoid organs, 2nd for challenge infections administered locally and not systemically and 3rd when the interval between challenge and tissue sampling is short (usually less than 5 days) (271, 273). As a rough approximation, the stringency of challenge experiments can be evaluated according to these rules. The VV-challenge measuring protection in the ovaries after 4-5 days, which we have used here, is thus a rather demanding assay, which needs a high level of protective immunity. However, different assays may well depend on particular qualities (i.e. pre-activation status) of the prevailing antigen specific cell population: peripheral challenges with pathogens that only replicate locally seem to favour conditions, where effector T cells or maybe effector memory T cells are present, since these cells express adhesion molecules, which are required for peripheral recirculation (222, 231, 236, 271). For protection against systemic challenges with pathogens that preferentially replicate within secondary lymphoid organs, the recirculation pattern might be of little relevance and the proliferative capacity of the pre-existing cell population may become more important, particularly if the interval between challenge and tissue sampling is rather long (228, 237, 265, 271).

From the available data it is clear, that central memory cells persist in the absence of antigen by homeostatic cytokine driven mechanisms, mainly IL-7 and IL-15 (274-276). Reencounter of antigen will lead to a second round of proliferation and effector cell differentiation followed by contraction and reversion first to effector memory and later to

central memory cells (237). However, it seems quite reasonable to speculate that the long-term maintenance of effector memory T cells is dependent on persisting or re-emerging antigen. Although our results support such a concept, direct experimental evidence is lacking.

As a synthesis of these opposing views we would like to propose, that maintenance of protective T cell mediated immunity is antigen dependent and probably mediated by effector memory cells, if the challenging antigen remains completely outside of secondary lymphoid organs. If the challenging antigen rapidly gains access to secondary lymphoid organs (i.v. challenge) and time for T cell expansion and differentiation is available, protective immunity seems to be antigen independent and efficiently provided by central memory cells. However, these experimental situations are two extremes of a continuum that rarely occur in nature. Therefore, maintenance of a balanced mixture of both memory cell populations is probably warranted for optimal protection. To achieve this without frequent boosting, persistent antigen is presumably an advantage.

According to another recent concept of T cell differentiation, termed 'programming' of T cells, an initial brief encounter of specific antigen in the order of 24-72h is sufficient stimulation for a naïve CD8⁺ T cell, to divide and differentiate into effector cells and subsequently into memory cells, without the need for additional antigen contact (277-280). During activation by initial antigen contact, a transcriptional program of the T cell is started, which leads to inheritable genetic changes, that drive the cell and her progeny along the line effector cell, effector memory cell and finally to the stage of a self renewing central memory cell with stem cell like properties (281). It has been postulated that even the contraction phase of an immune response is programmed (220). However,

the quantity and quality of the ensuing effector and memory cell populations depends on a variety of exogenous factors during the whole differentiation pathway including molecular factors (avidity between TCR and MHC-peptide complexes, density of MHC-peptide, duration of their interaction, costimulation, cytokine and chemokine environment), cellular factors (nature, origin and activation of APC, CD4⁺ T cell help) and anatomical factors (localisation of initial interaction, i.e. secondary lymphoid organ vs. periphery) (281). Overall, full activation of naïve T cells only occurs efficiently in secondary lymphoid organs, where these interactions take place in a timely and anatomically coordinated fashion (162, 282).

It is very fascinating to study ‘programming’ of T cells without the confounding influence of further antigen contact and to demonstrate that initial events have such profound downstream effects. However, since most of these factors are crucially influenced by antigen load, density, distribution and persistence, these models do not reflect natural situations. Although it is clear that high level persistence of antigen has a detrimental influence on the maintenance and functionality of memory CD8⁺ T cells (168, 283), the tendency to interpret these recent results on ‘programming’ of T cells in the sense, that any level of persistent antigen generally has a negative influence on immunity (229, 272), is ignorant of a large body of older immunological evidence (267, 270).

Other recent studies of memory T cell differentiation have identified certain markers including expression of the IL-7 receptor (R) α -chain and of CD8 $\alpha\alpha$ homodimers on a subset of effector cells, that preferentially give rise to long term memory cells, whereas the majority of other effector cells undergo apoptosis during the contraction phase of an

immune response (284, 285). By persistent high level expression of the IL-7R α these cells survive independent of further antigenic stimulation during the memory phase by IL-7 and possibly IL-15 driven homeostatic proliferation (274-276).

In addition, several research groups have recently demonstrated the profound influence of CD4⁺ T cells on the development of a robust memory CD8⁺ T cell response. For many years it was speculated, that early help by CD4⁺ T cells might be crucial for efficient CD8⁺ T cell responses in viral infections with the potential for high level persistence (144, 170, 171, 286). Now, more experimental evidence is emerging and potential mechanisms have been proposed: an efficient primary response of CD8⁺ T cells develops in the absence of CD4⁺ T cells but the generation of CD8⁺ T cell memory is greatly impaired mainly by a poor proliferative response to secondary challenge (174-177). It remains to be elucidated what the exact mechanism of the interaction between CD4⁺ and CD8⁺ T cells is and how the important signals are transferred. A direct cognate interaction seems to be unlikely, since murine CD8⁺ T cells do not express MHC class II. Conflicting data have been generated, whether the interaction between CD40 (expressed on CD8⁺ T cells) and CD40L (expressed on CD4⁺ T cells) is crucially involved to provide help in this setting, similar to the interaction between B cells and T helper cells (174, 287, 288). However, soluble mediators may also be involved but their individual contribution has not been evaluated so far.

These studies have important implications both for the understanding of the pathogenesis of persistent infections and for rational vaccine design. In persistent infections the role and the function of CD4⁺ T cells needs to be carefully re-assessed together with the phenotype and function of the ensuing CD8⁺ T cell response, particularly during the

earliest events of primary infection. It will be very difficult to perform such analysis in human diseases, but in primary HIV- and EBV-infection such studies might shed light on mechanisms of early pathogenesis. In HIV-infection, preferential infection of HIV-specific CD4⁺ T cells has been demonstrated (145) but it is unclear, whether this is responsible for a rapid loss of helper activity for CD8⁺ T cells, which then develop functional impairment. Different CD8⁺ T cell epitopes could be affected differently and it would be interesting to analyse, whether linked T cell help (a CD4⁺ T cell response targeting an epitope derived from the same viral protein) has an influence on the functionality of CD8⁺ T cells. In EBV-infection, information could be gathered concerning the mechanism behind the massive culling of certain responses, while others remain much less affected (127), particularly if a differential expression of IL-7R during the expansion phase could be demonstrated. In acute EBV-infection, lack of specific CD4⁺ T cell help seems unlikely but 'programming' of contraction will not be accessible for analysis *in vivo*.

In MCMV-infection, we observed clearly less contraction of the CD8⁺ T cell response after primary infection compared to other acute viral infections. It will be extremely interesting, to analyse the level of IL-7R α expression and the proliferative potential of MCMV-specific CD8⁺ T cells longitudinally, particularly if separately analysed for effector memory and central memory cells.

In addition, the MCMV-specific CD8⁺ T cell response in the absence of CD4⁺ T cells or in the absence of CD40/CD40L-interaction needs to be analysed longitudinally, since older studies suggest that CD4⁺ T cells are crucial for complete clearance of productive MCMV-replication in salivary glands (96, 97). However, it needs to be re-assessed whether this is a direct antiviral effect or whether CD4⁺ T cells mainly influence other

effector cells. Moreover, failure of newborns and young children to terminate productive HCMV-replication in salivary gland and kidney was associated with a functional defect of CD4⁺ but not CD8⁺ T cells (58, 101). Therefore, these studies of 'help-deficient' mice need to (re-) address, 1) whether clearance of primary MCMV-infection and establishment of latency is clearly impaired, 2) whether the frequency (inflation ?), phenotype and function of CD8⁺ T cells is affected by repetitive or continuous encounter of low levels of antigen in the absence of CD4⁺ T cells and 3) whether strategies could be developed to rescue functionally impaired CD8⁺ T cells in the setting of a persistent viral infection (if there is evidence for functional impairment).

For rational design of T cell based vaccines, these studies have profound implications as well: the concept of 'programming' would suggest that everything is decided during the first 24-72 hours. If the initial antigen contact is optimally tuned, then further antigenic stimulation is not needed for protective immunity. CD4⁺ T cells might just be one of the factors needed for optimal 'programming'. However, a most recent study by Sun et al contradict this concept (289). They have shown that CD4⁺ T cells were needed for the antigen independent maintenance of memory CD8⁺ T cells but not for their 'programming'. Overall, induction of CD4⁺ T cells seems to be beneficial for the generation of protective immunity mediated by CD8⁺ T cells, but the exact timing and mechanisms of help remain poorly defined.

According to the study of Wherry et al, the interval between priming and boosting needs to be carefully evaluated to ensure optimal efficacy, since central memory cells only will proliferate maximally upon boosting (237). However, these latter results need confirmation as outlined previously.

Probably, truth lies between the extremes of opinions and might in addition be different for every individual host-pathogen-situation: neither an initial 'super-boost' nor a constant low level tickling of antigen will be optimal for protective immunity. The optimal regimen for a T cell based vaccine will require a very solid initial priming event with optimal T cell help and a careful dosing and timing of boosting possibly by different vectors and repetitively. Such a delicate balance between antigen and immune system of the host will be difficult to find and additional questions need to be addressed concerning epitope targeting (frequency of cells and breadth of a response), use of adjuvans or of live attenuated vectors and maybe even use of persistent vectors (267). The more we learn about the basic rules regulating the interactions between molecules, cells, organs and whole organisms (particularly pathogen and their hosts), the better and more rational will be our efforts to generate successful vaccines for diseases, where we have failed so far. Our studies on MCMV-infection of mice highlight one possible outcome of a particular host-pathogen interaction. We think, that further studies should be undertaken to evaluate, whether this specific interaction leading to memory inflation, can be used for the benefit of the host.

6. Figure legends

Figure 1: Accumulation of pp89-specific CD8⁺ T cells after infection with MCMV:

(A) MCMV-titre: Mice were infected with 10⁶ PFU MCMV i.v. and at indicated time points MCMV-titres were determined in spleen (filled circles) and salivary gland (open circles) by virus plaque assay (lung and liver not shown). MCMV-titres represent log₁₀(PFU/organ). Each line represents the mean of 3-4 mice per time point. Error bars indicate the standard deviation (SD). Detection limits of the assays: spleen: 1.6 (40 PFU/organ), salivary gland: 2.0 (100 PFU/organ).

(B) pp89-specific CD8⁺ T cells in blood: Mice were infected with 10⁶ PFU MCMV (filled diamonds) or 5x10⁶ PFU Vac89 (filled circles) i.v. At the indicated time points blood was taken and PBL were stained with pp89-tetramer and anti-CD8. The percentage of CD8⁺ T cells staining with pp89-tetramer is plotted over time. Each line represents the mean of 3-4 mice per experimental group. Error bars indicate the SD within experimental groups.

(C and D) pp89-specific CD8⁺ T cells in the spleen: At indicated time points spleen cells were harvested and the frequency of pp89-specific T cells was determined by pp89-tetramer staining (MCMV: filled diamonds, Vac89: filled circles) and by pp89-IFN γ (MCMV: open diamonds, Vac89: open circles). In C, the percentage of CD8⁺ T cells positive for pp89-tetramer or pp89-IFN γ is plotted over time and in D, the total number of pp89-specific CD8⁺ T cells per spleen is depicted (number x 10⁵). Each line represents the mean of 3-4 mice per experimental group. Error bars indicate the SD within an experimental group.

(E) FACS[®] dot plots from mice 10 and 400 days after MCMV-infection, from mice 6 and 300 days after Vac89-infection and from naïve mice. Panels were gated on live lymphocytes without B cells (excluded by anti-CD45R-staining). Numbers indicate the mean percentage of pp89-tetramer⁺ or IFN γ -producing CD8⁺ T cells of 3-4 mice per group.

Day 300 and day 400 are data from a single experiment of 4 mice per group. Data were confirmed by two similar experiments extended until day 250 after infection.

Figure 2: (A) Evolution and distribution of pp89-specific T cells in different organs:

Balb/c mice were infected with MCMV (1×10^6 PFU i.v.). Ten, 40 or 230 days after MCMV infection lymphocytes were isolated from different organs and stained with the pp89-tetramer and anti-CD8. The percentage of tetramer positive CD8⁺ T cells is shown for each organ. The left panel shows data from lymphoid organs and blood, the right panel from non-lymphoid organs. Note the difference in scale. Columns represent the mean of 3 mice per group; error bars indicate the SD within an experimental group. One out of three similar experiments are shown.

(B) Increasing oligoclonality of pp89-specific CD8⁺ T lymphocytes over time:

A panel of TCR V β reactive antibodies were used to identify V β usage by pp89-specific T cells over time in the spleen (day 9, 89, 272 post infection). The columns show the percentage of pp89-specific T cells positive for the indicated V β -family. V β 8.1-specific Abs also detect V β 8.2 positive cells. 3-4 mice were used per experimental group. Error bars indicate the SD within an experimental group. One out of two representative experiments are shown.

Figure 3: Phenotypic characterization of pp89-specific CD8⁺ T cells after infection with MCMV or Vac89:

Mice were infected with 10⁶ PFU MCMV, with 5x10⁶ PFU Vac89 i.v. or left uninfected as a naïve control. Spleen cells were harvested at the indicated time points and stained with pp89-tetramer, anti-CD8, anti-CD45R and with the indicated phenotypic marker. Panels are gated on live CD8⁺ lymphocytes. Numbers indicate the mean of 3-4 mice per group and represent the mean percentage of pp89-tetramer⁺ CD8⁺ T cells (row 1-4) or of naïve CD8⁺ T cells (row 5) expressing the respective marker. One out of four (day 6 and 10) similar experiments are shown. Results from day 300 and day 400 were confirmed by two comparable experiments from 160 and 230 days after infection.

Figure 4: (A) CD69 expression by pp89-specific T cell during MCMV latency in different organs:

Lymphocytes of mice infected with MCMV 230 days previously were isolated from different organs and stained with pp89-tetramer, anti-CD8 and anti-CD69. The percentage of pp89-tetramer positive CD8⁺ T cells expressing CD69 in each organ is plotted. Columns represent the mean of 3 mice and error bars indicate the SD. One out of two comparable experiments are shown.

(B) In vivo proliferation of pp89-specific CD8⁺ T cells after MCMV infection:

MCMV infected mice (day 0 and day 220) were treated with BrdU for 10 days. Splenocytes were then isolated and stained with pp89-tetramer, anti-CD8 and anti-BrdU antibodies or an isotype control. Panels are gated on CD8⁺ T cells. Numbers indicate the % of tetramer positive cells, which are BrdU positive. Staining with an isotype control Ab is shown on the left. In the middle, BrdU-incorporation is shown 10 days after

infection and on the right 230 after infection. One out of 3 mice are shown. The experiment was repeated three times with similar results.

Figure 5: Long-term accumulation of memory cells is not restricted to pp89-specific CD8⁺ T cells:

Balb/c mice were infected with 10^6 PFU MCMV i.v. Spleen cells were taken at different time points thereafter and were tested *ex vivo* by pep-IFN γ . Permeabilised cells were stained with anti-IFN γ and anti-CD8 antibodies and the mean percentage of IFN γ -producing CD8⁺ T cells of 3-4 mice per group is plotted. Error bars indicate the SD within an experimental group. The following MCMV-derived peptides from 5 different viral proteins were used (MHC class I presenting molecule): pp89 (H2-L^d), m04/gp34 (H2-D^d), M84/p65 (H2-K^d), M83/pp105 (H2-L^d) and m164 (H2-D^d). (A) Frequency of IFN γ producing T cells specific for the immunodominant epitopes pp89 and m164. (B) Frequency of IFN γ producing T cells specific for subdominant epitopes derived from m04, M83 and M84. Note the 10-fold difference in scale between panel C compared to A and B. One out of three comparable experiments are shown.

Figure 6: Protective immunity mediated by pp89-specific CD8⁺ T cells is dependent on the dose of infection and the timing of challenge:

BALB/c mice were infected with 10^6 (open diamonds) and 10^3 (filled diamonds) PFU MCMV i.v. or were left naïve (open circles) as a negative control. Thereafter, the pp89-specific CD8⁺ T cell response was measured longitudinally in the blood (Fig. 6A). The percentage of CD8⁺ T cells staining with pp89-tetramer is plotted over time. Each line

represents the mean of 4-5 mice per experimental group. Error bars indicate the SD within experimental groups.

At indicated time points after MCMV-infection, mice were challenged with Vac89 i.p. and the VV-titres were measured in the ovaries 4 days later (Fig. 6B). Titres are given as $\log_{10}(\text{PFU Vac89/ovaries})$. The detection limit (det. lim.) and the SD are indicated. Each line represents the mean of 4-5 mice per experimental group. One out of two similar experiments are shown.

To measure the expansion of specific T cells after challenge the frequency of pp89-specific CD8^+ T cells was determined by tetramer-staining before and 4 days after challenge with Vac89 in the spleen at indicated time points after MCMV-infection (Fig. 6C, blood, liver, and ovaries not shown). The percentage of CD8^+ T cells staining with pp89-tetramer is plotted over time. Each line represents the mean of 3-4 mice per experimental group. Error bars indicate the SD within experimental groups.

Figure 7: Efficient induction of specific CD8^+ T cells after infection with recombinant MCMV:

7A: Western-blot analysis of expression of recombinant proteins from viruses in vitro. MEF were infected with wild type (wt) MCMV, with MCMV-NP (NP), with MCMV-GP (GP) or, as a negative control, with a mutant MCMV lacking expression of IE2 (Δie2). To make IE2 expression detectable, protein transcription and translation was synchronized by treatment of cells with Cycloheximide for the first 3 hours of infection and with Actinomycin D 4 to 7 hours post infection. Protein expression was detected 7 hours post infection by a mAb specific for IE2. The appropriate IE2 bands are marked on

the figure. The NP-IE2 and GP-IE2 fusion proteins show a slightly higher molecular weight due to the additional NP and GP epitopes, respectively.

7B: C57BL/6 mice were infected with 2×10^6 PFU MCMV-GP (left panel) or MCMV-NP (right panel). Ten days after infection, splenocytes were harvested, restimulated for 5 days in vitro and then tested in a $5\text{h-}^{51}\text{Cr}$ -release assay on target cells pulsed with GP33 (filled triangles), NP366 (filled squares) or an irrelevant peptide (open circles). Each line represents an individual mouse. One out of 4 experiments are shown.

7C: NP-tetramer staining of spleen cells from a mouse infected 10 days previously with MCMV-NP. The upper panel shows an ex vivo tetramer staining. For the lower panel, splenocytes from the same mouse were restimulated for 7 days in vitro with NP366-pulsed naïve spleen cells. Numbers indicate the percentage of NP-specific cells of CD8^+ T cells. Panels are gated on live lymphocytes without B-cells. One out of 7 mice are shown.

Figure 8: Phenotypic characterization of NP-specific CD8^+ T cells after infection with MCMV-NP or influenza:

C57BL/6 mice were infected with 2×10^6 PFU MCMV-NP i.v. or with 100 hemagglutination units (HAU) influenza i.n. Spleen cells were harvested at the indicated time points and stained with NP-tetramer (NP-tet), anti-CD8, anti-B220 and with anti- $\text{CD43}^{\text{IB11}}$ or anti-CD62L. Panels are gated on live lymphocytes without B-cells (Fig. 8A) or on live CD8^+ lymphocytes (Fig. 8B & 8C). Numbers indicate the percentage of NP-specific cells of CD8^+ T cells (Fig. 8A) or the percentage of NP-specific CD8^+ T cells expressing $\text{CD43}^{\text{IB11}}$ (Fig. 8B) or CD62L (Fig. 8C). One out of 3 similar experiments are shown.

Figure 9: Increasing protective CD8⁺ T cell -mediated immunity after infection with MCMV-NP:

C57BL/6 mice were infected with 2×10^6 PFU MCMV-NP i.v. (filled squares) or with 100 HAU influenza i.n. (filled inverted triangles) or they were left naïve (open circles) as a control. Thereafter, the NP-specific CD8⁺ T cell response was measured longitudinally in the blood (Fig. 9A). The percentage of CD8⁺ T cells staining with NP-tetramer is plotted over time. Each line represents the mean of 4-5 mice per experimental group. Error bars indicate the SD within experimental groups.

At indicated time points after infection with influenza (Fig. 9B) or MCMV-NP (Fig. 9C), mice were challenged with VacNP i.p. and the VV-titres were measured in the ovaries 4 days later (as in Fig. 6B). Titres are given as $\log_{10}(\text{PFU VacNP/ovaries})$. The detection limit (det. lim.) and the standard deviation (SD) are indicated. Each line represents the mean of 4-5 mice per experimental group. The experiment was repeated with comparable results until day 220.

Figure 10: Increasing protective CD8⁺ T cell -mediated immunity after infection with MCMV-GP:

C57BL/6-mice were either infected with 2×10^6 PFU MCMV-GP i.v. (filled triangles), with 5×10^6 PFU VacGP i.v. (open triangles) or immunized with the peptide GP33 and CFA s.c. (filled diamonds) or they were left naïve (open circles) as a control. Thereafter, the GP-specific CD8⁺ T cell response was measured longitudinally in the spleen by ICS (Fig. 10A). The percentage of CD8⁺ T cells staining for intracellular IFN γ after

stimulation with GP33-41 is plotted over time. Each line represents the mean of 3-4 mice per experimental group. Error bars indicate the SD within experimental groups.

At indicated time points after immunization with GP33+CFA (Fig. 10B, upper panel) or after infection with MCMV-GP (Fig. 10B, lower panel), mice were challenged with VacGP i.p. and the VV-titres were measured in the ovaries 4 days later. Titres are given as $\log_{10}(\text{PFU VacGP/ovaries})$. The detection limit was 1.6 and the SD is indicated. Each line represents the mean of 4-5 mice per experimental group.

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8. List of Abbreviations

BAL	broncho-alveolar lavage	MCMV-GP	MCMV recombinant for
BM	bone marrow		LCMV-GP
BrDU	Bromo-deoxy-uridin	MCMV-NP	MCMV recombinant for
CMV	cytomegalovirus		Flu-NP
E	early	MEF	mouse embryonic
EBV	Epstein-Barr Virus		fibroblast
Flu-NP	nucleoprotein of influenza	MHV-68	murine γ -Herpesvirus-68
GP	glycoprotein	NP	nucleoprotein
HCMV	human cytomegalovirus	pep	peptide
HIV	human Immunodeficiency	pp	phosphoprotein
	Virus	tetramer	MHC class I tetrameric
ICS	intracellular cytokine		complexes
	staining	V β	variable region of T cell
IE	immediate-early		receptor β -chain
Influenza	Influenza A virus	VV	Vaccinia virus
L	late	Vac89	VV recombinant for pp89
LCMV	lymphocytic chorio-	VacGP	VV recombinant for
	meningitis virus		LCMV-GP
LCMV-GP	glycoprotein of LCMV	VacNP	VV recombinant for
LN	lymph node		Flu-NP
MCMV	murine cytomegalovirus	VZV	Varicella Zoster virus

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Table 1: Total number of MCMV specific CD8⁺ T cells in the spleen over time.

	Day p.i.	Total number of epitope specific CD8 ⁺ T cells/spleen (x10 ⁴)			
		IE1/pp89	m04	M83	M84
MCMV	8	143±36	16±6	6±1.7	6±3.8
	32	82.5±2	2.5±0.8	2±0.8	1.5±0.5
	400	150±21	1.1±0.2	1.8±0.9	1.2±0.2
Vac89	6	103±38	n.d.	n.d.	n.d.
	28	16±4			
	300	7.1±0.3			

Data are derived from the experiments shown in Fig 1 and Fig 5 (6-8 mice per group). Numbers represent the mean ± the standard deviation within the group of mice. Total counts of spleen cells were determined and virus-specific T cell frequencies were calculated for every single MCMV-derived epitope using the data from ICS. The frequencies for m164 specific cells (which were analysed in a separate set of experiments) showed frequencies similar to those of pp89 specific cells (as shown Fig 5A and 5B).

Table 2:
Total number of epitope-specific CD8⁺ T cells per spleen at different time points after infection with MCMV, influenza or after immunization with peptide GP33.

epitope	Total number of epitope-specific CD8 ⁺ T cells / spleen (x 10 ⁴)					
	IE1/pp89		NP366			GP33
	MCMV (10 ⁶)	MCMV (10 ³)	MCMV-NP	Influenza	MCMV-GP	GP33+CFA
immunization						
Days p.i.						
8-11	56.8 ± 20	2.4 ± 0.3	2.5 ± 1.1	55.8 ± 28.5	1.1 ± 0.5	25.1 ± 9.7
18-22	31.3 ± 8.7	2.9 ± 0.8	2.9 ± 1.6	20.3 ± 10	2.0 ± 0.7	
35-45	26.5 ± 14.4	7.9 ± 2.8	6.9 ± 3.7	4.8 ± 0.9	10.4 ± 3.7	18.7 ± 6.4
100-160	59 ± 13.1	24.5 ± 6.9	12.7 ± 4.8	5.2 ± 2.2	15.5 ± 6.3	10.9 ± 2.6
250-350	115 ± 28	45.8 ± 22	9.2 ± 5.3	3.9 ± 0.5		

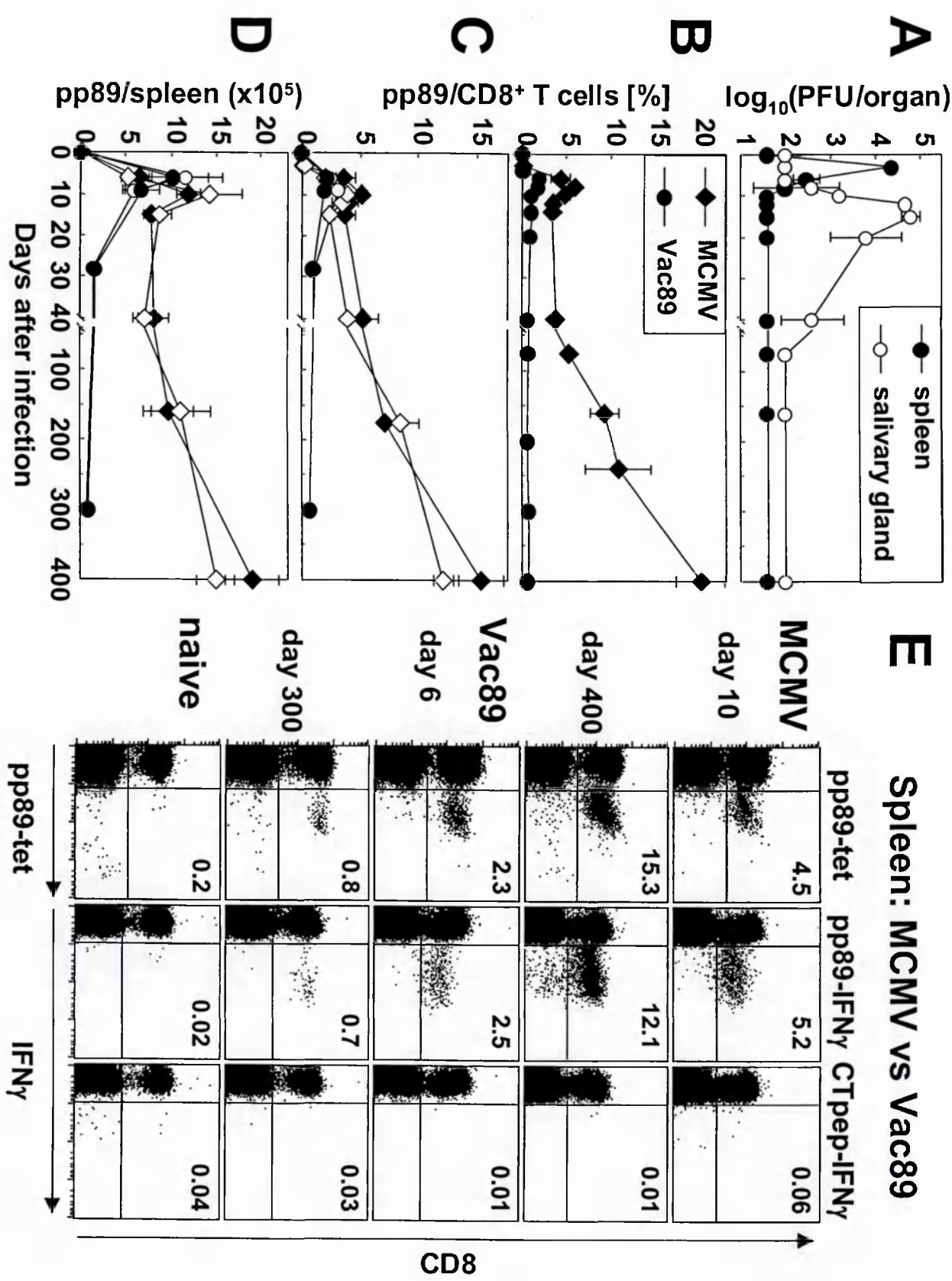
Mice were infected or immunized with the indicated virus or antigen. At different time points thereafter the total number of epitope-specific CD8⁺ T cells per spleen was measured by FACS using tetramer staining (column 1-4) or ICS (column 5 & 6). Mean numbers ± standard deviation are given for 4-12 mice per group. Numbers should be multiplied by 10⁴. Data from 1-3 experiments with the same antigen/virus were pooled for calculation. 10⁶ and 10³ indicates the virus dose in PFU used for infection. Days p.i. stands for days after infection.

Table 3:
Correlation between the frequency of epitope-specific CD8⁺ T cells, the level of protection measured by VV-challenge and the time after priming with MCMV-wt, MCMV-NP or Influenza.

Correlation of <i>a</i> versus <i>b</i> (Spearman's rho)				
	<i>a</i>	Frequency of specific cells	Frequency of specific cells	Time after priming
	<i>b</i>	Time after priming	Level of protection	Level of protection
MCMV (10 ³)	n=40	0.841, <i>p</i> <0.001	0.521, <i>p</i> =0.001	0.483, <i>p</i> =0.002
MCMV-NP	n=23	0.702, <i>p</i> <0.001	0.507, <i>p</i> =0.014	0.513, <i>p</i> =0.012
Influenza	n=23	- 0.882, <i>p</i> <0.001	0.760, <i>p</i> <0.001	- 0.736, <i>p</i> <0.001
Overall	n=86		0.566, <i>p</i> <0.001	

Mice were primed with the indicated virus and the frequency of epitope-specific CD8⁺ T cells was monitored in the blood by tetramer staining. Frequencies used for calculation were measured 0-3 days before challenge. Four days after challenge VV-titers were measured in the ovaries. The level of protection was calculated according to materials and methods to correct for variation between different challenge experiments. Numbers indicate the correlation coefficients which were calculated with Spearman's rank correlation using logarithmic scales for all variables. Two tailed p-values for each correlation are indicated in the table. Data from 1-3 experiments per virus and time point were included into the analysis.

Fig. 1



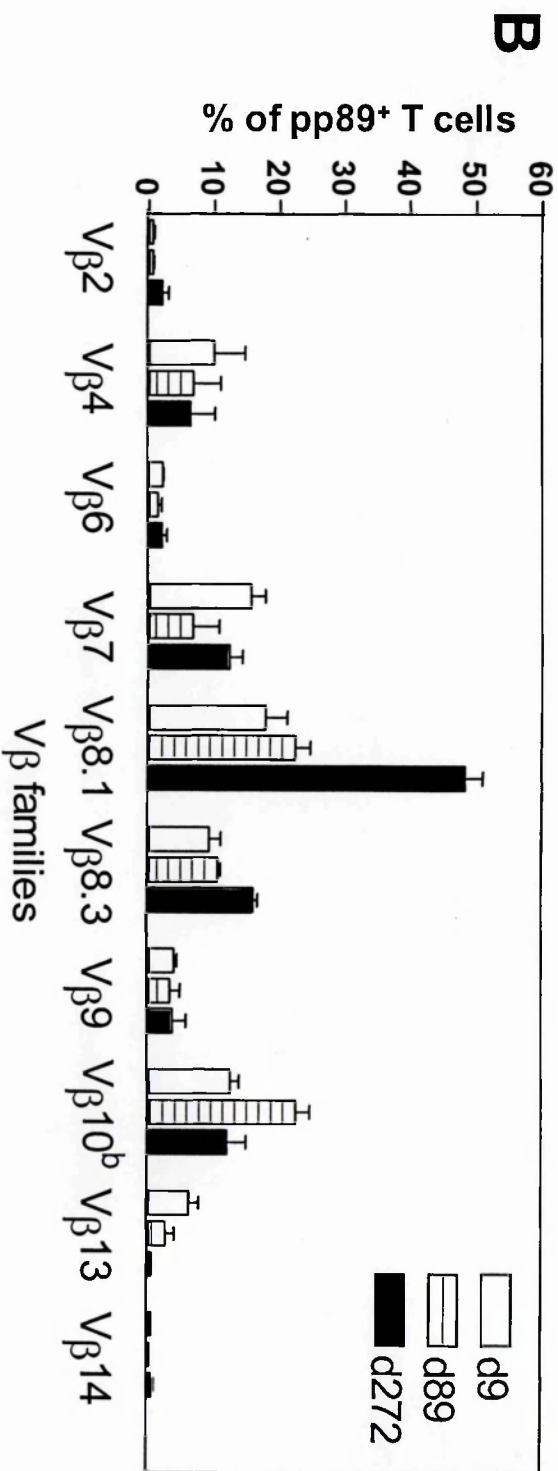
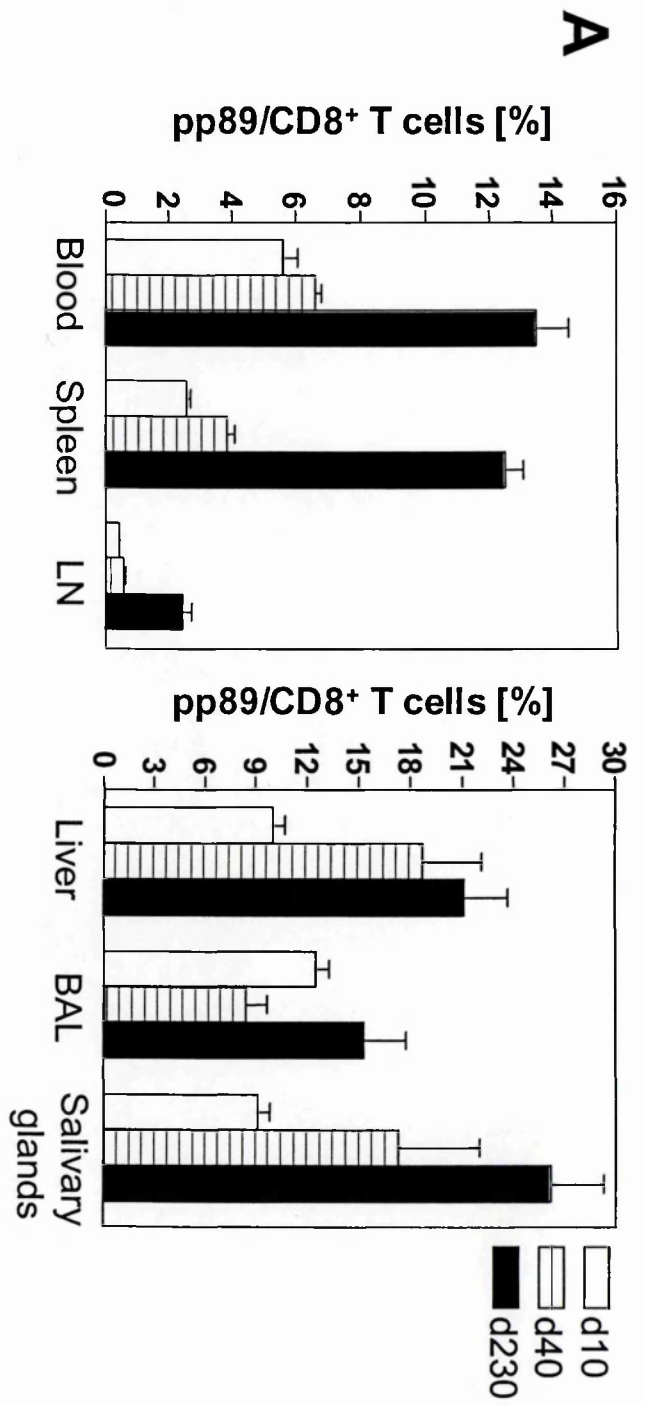


Fig. 2

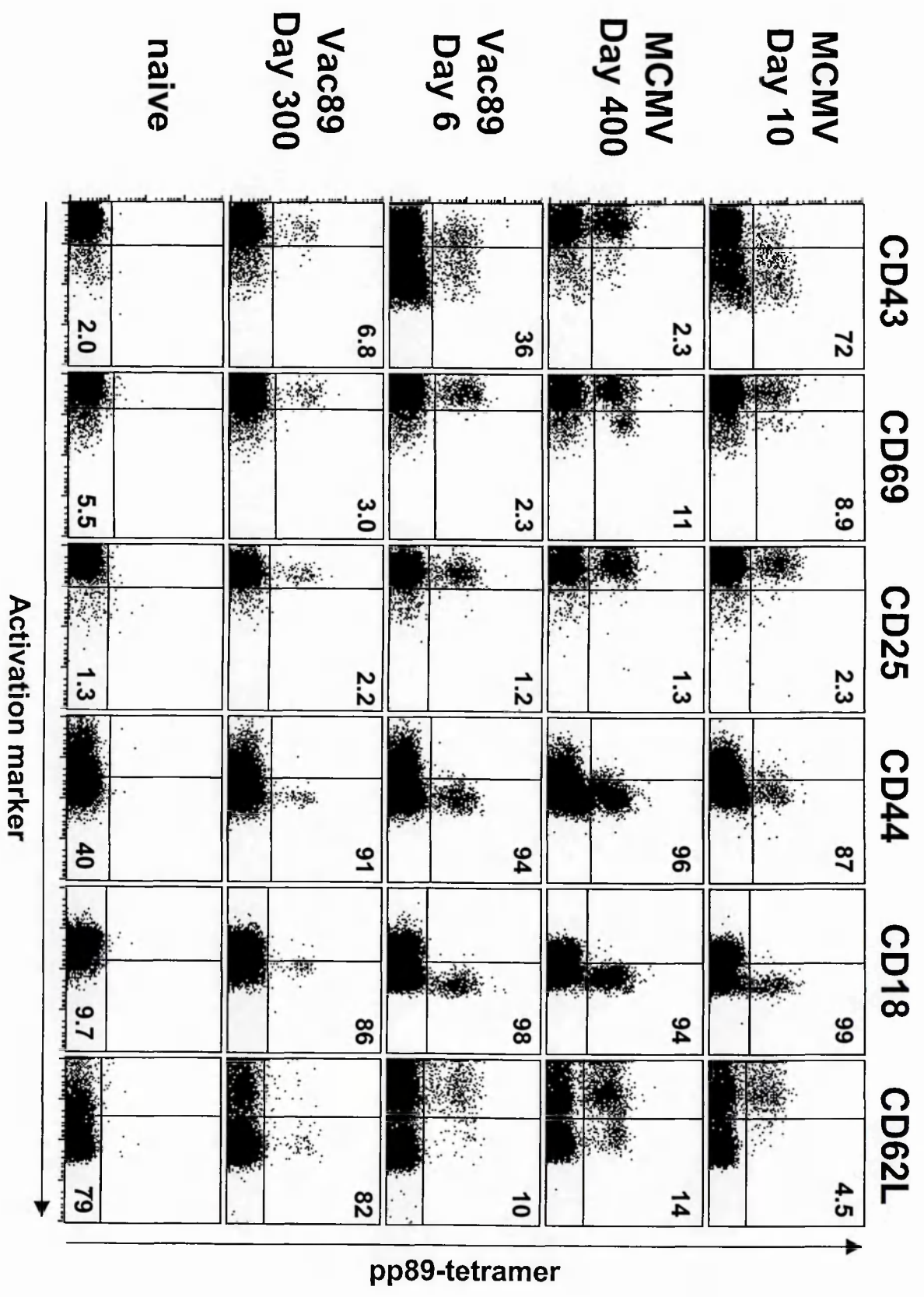


Fig. 3

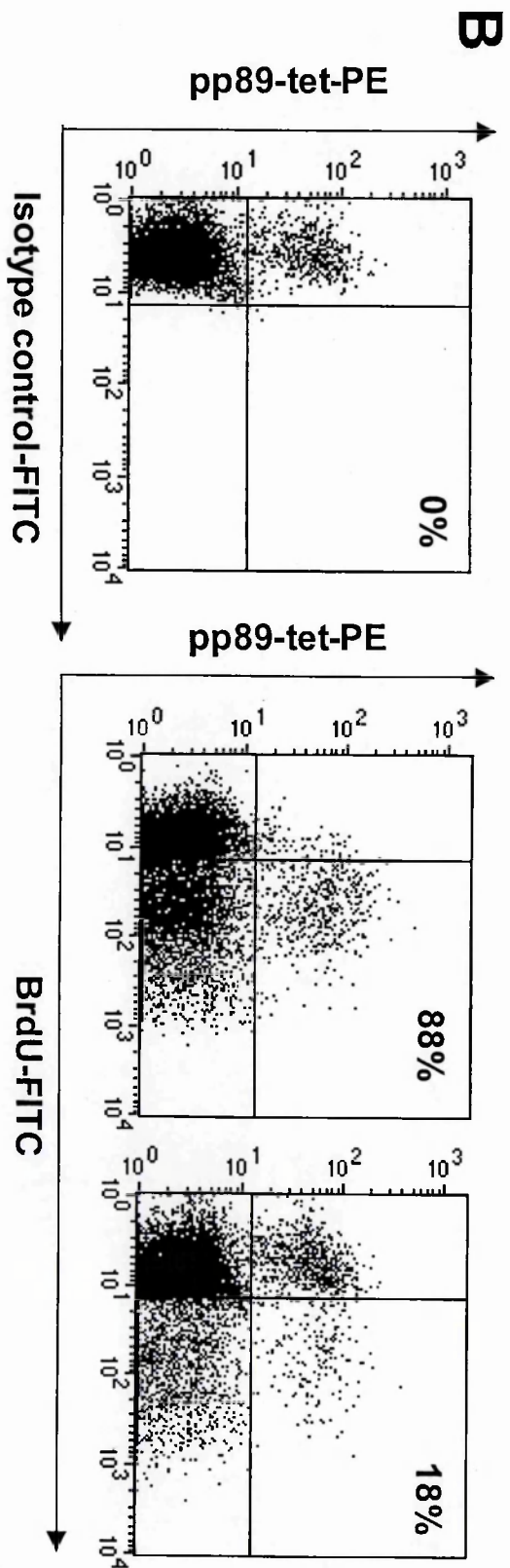
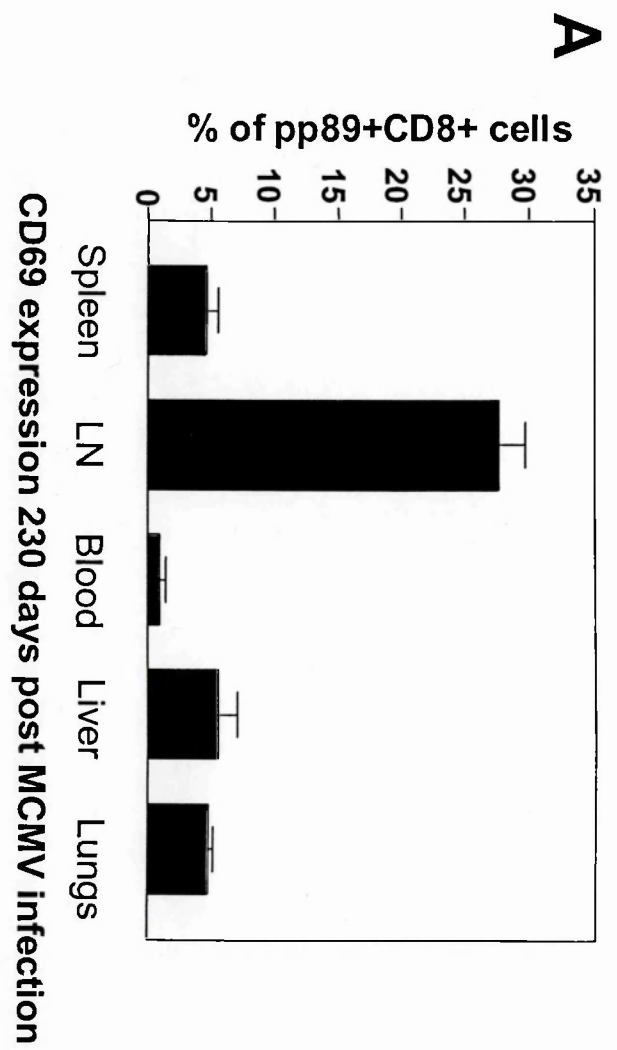


Fig. 4

Fig. 5

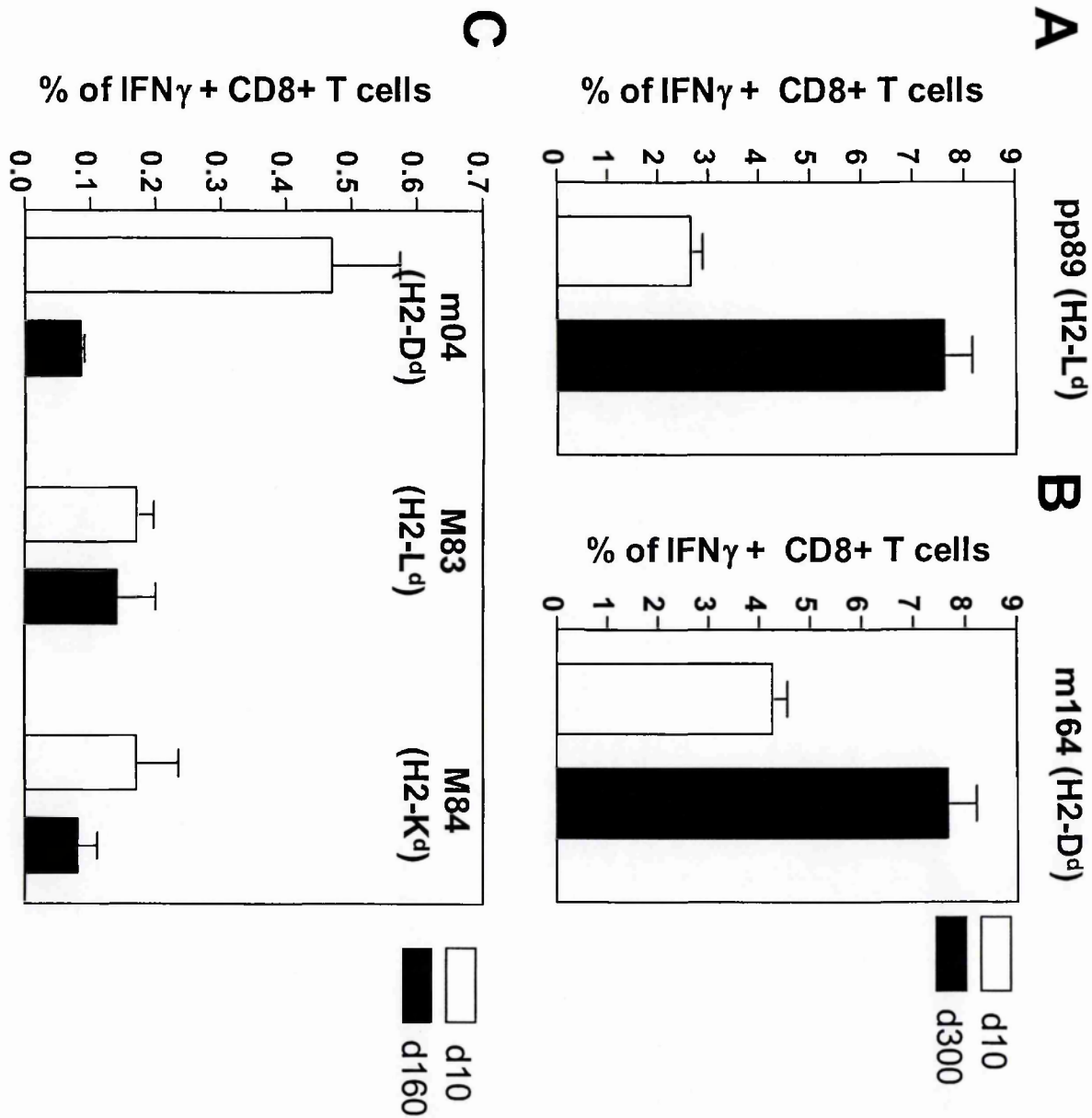


Fig. 6

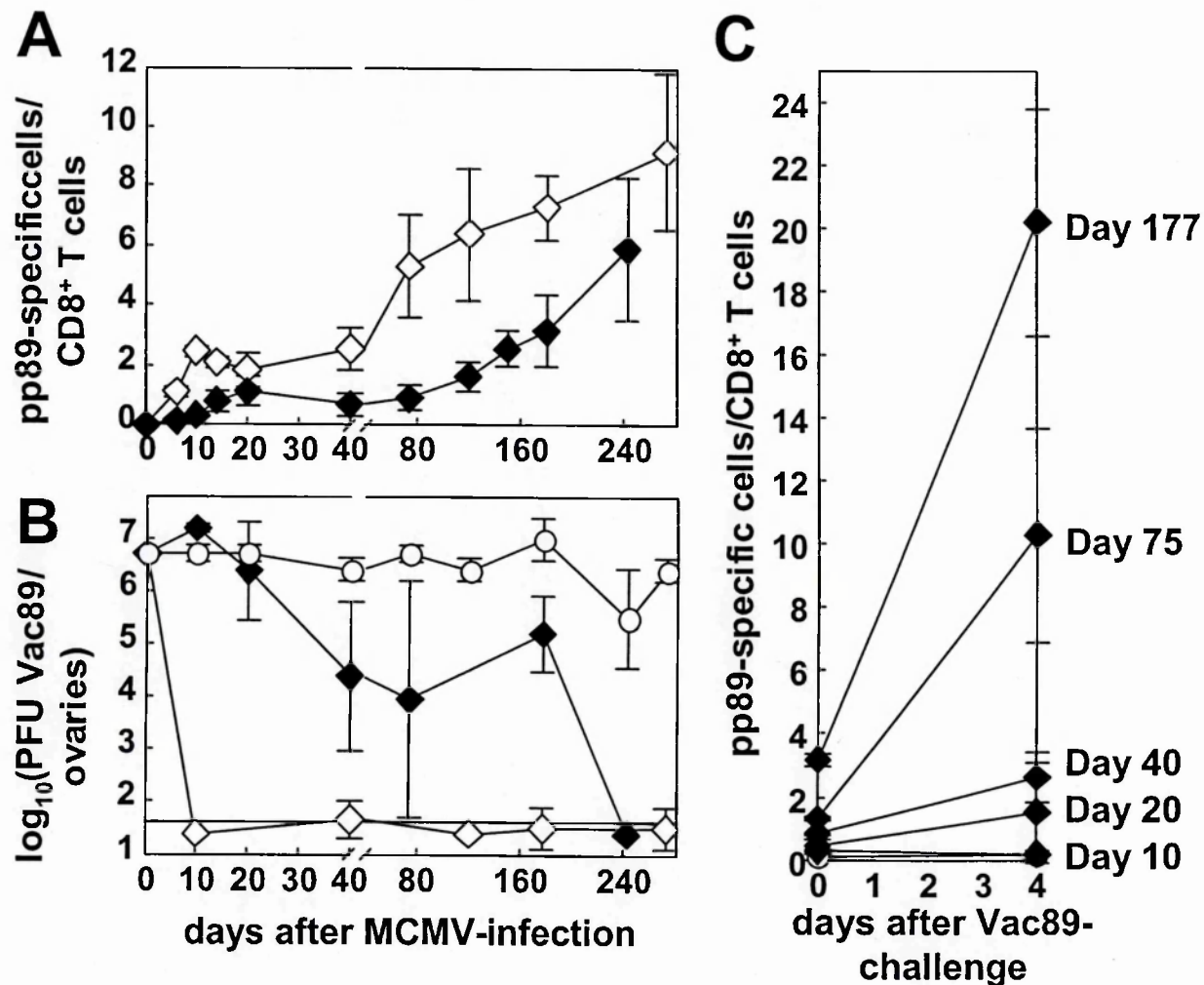


Fig. 7

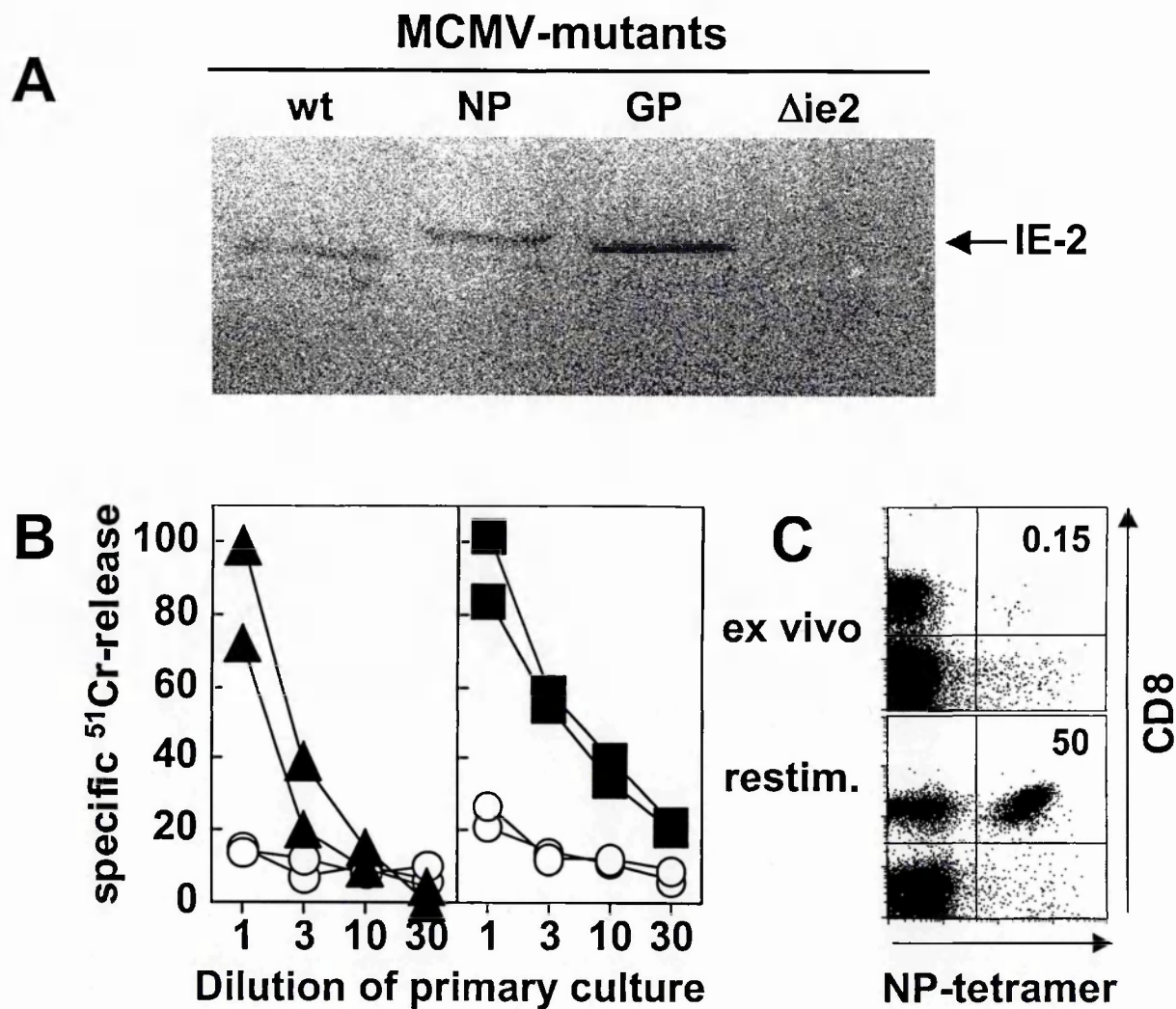


Fig. 8

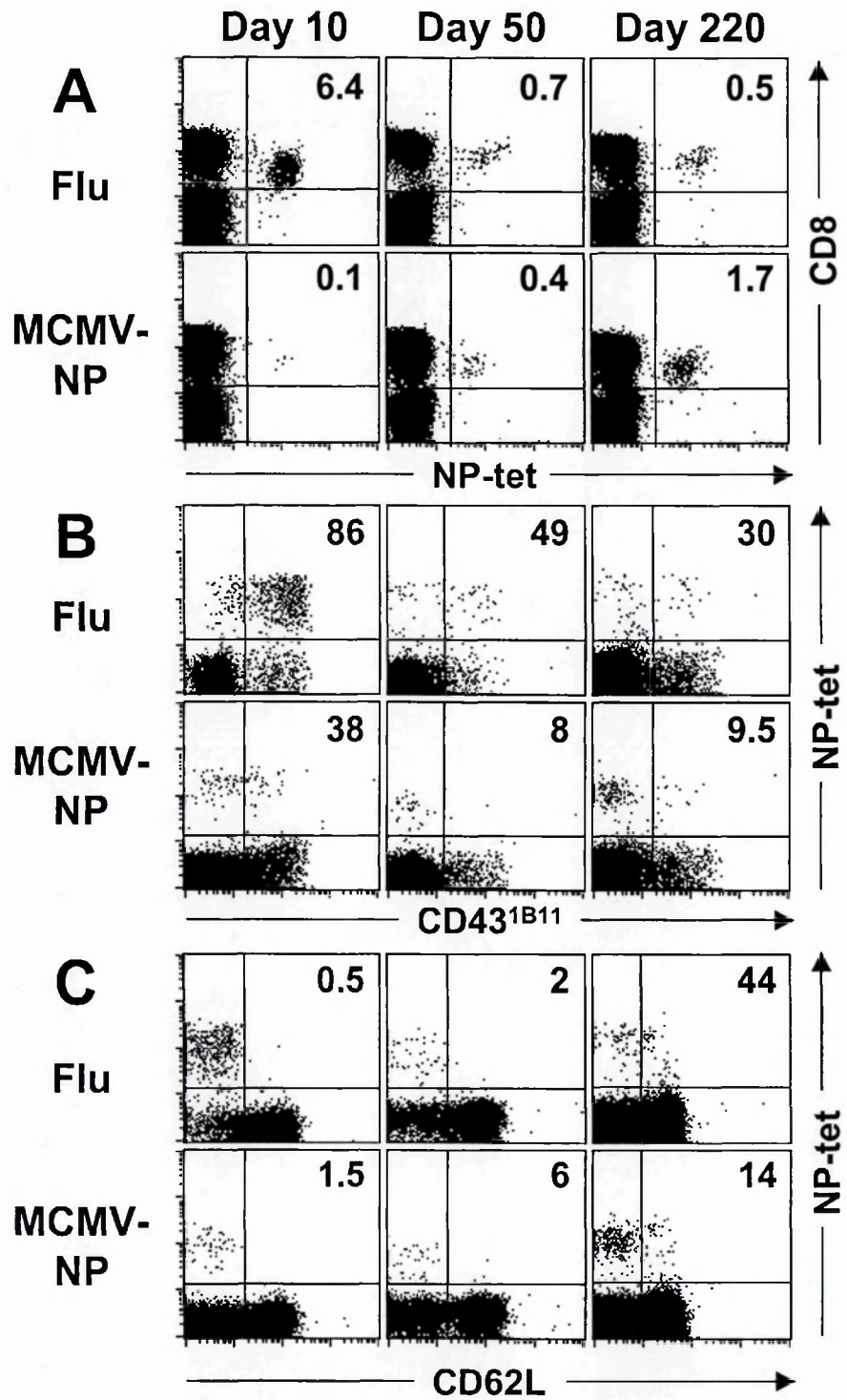


Fig. 9

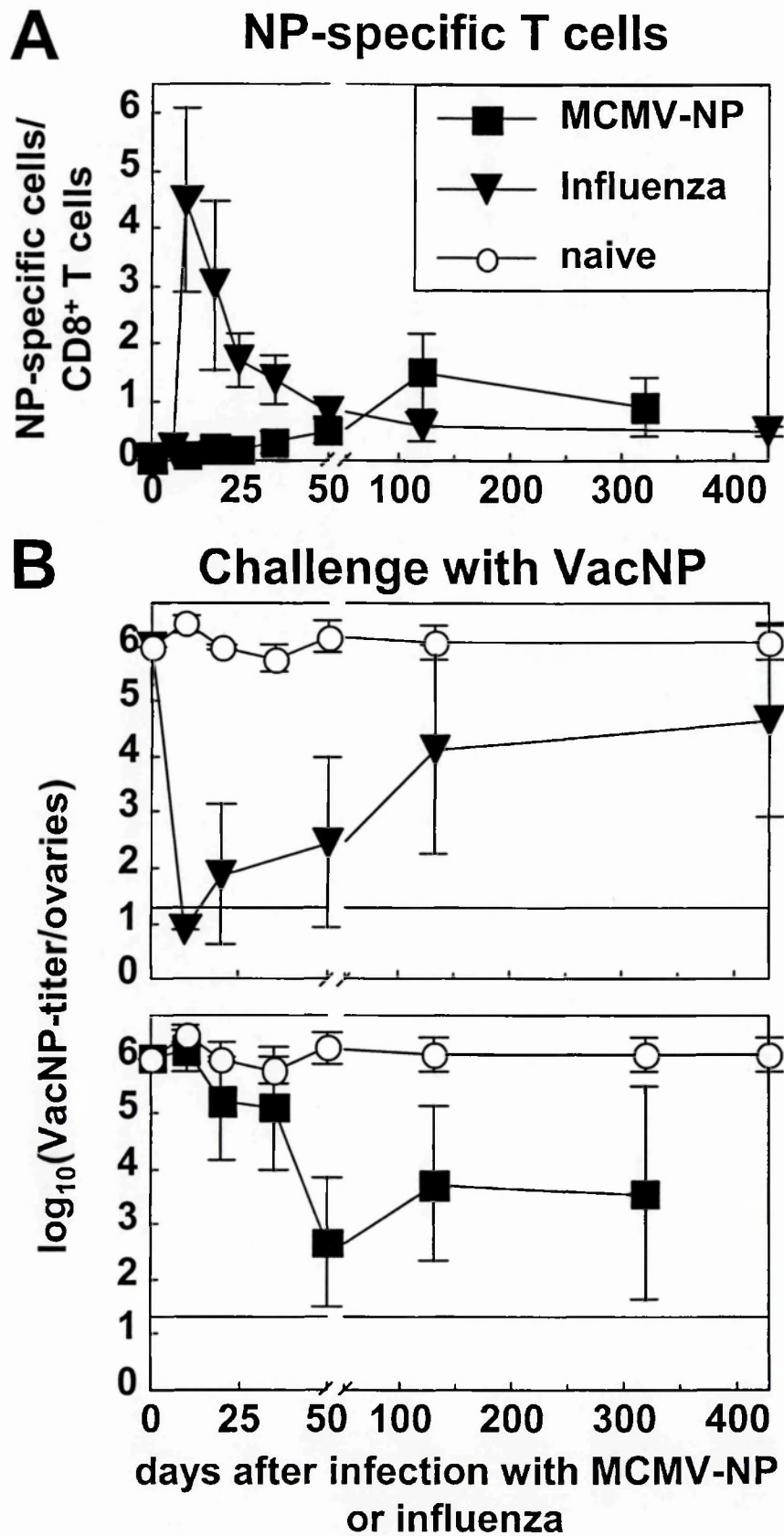


Fig. 10

